Genetic and serologic characterization of a Swedish human hantavirus isolate

Marie Lindkvist

Umeå 2008
To my family
Abstract

Hantaviruses are found practically all over the world and cause hemorrhagic fevers in man. Each year about 150,000 people are hospitalized in these zoonotic infections which can be of two types: hemorrhagic fever with renal syndrome (HFRS) or hantavirus cardiopulmonary syndrome (HCPS), depending on the infecting virus. Hantavirus infections are emerging infectious diseases. That is, the number of reported cases of hantaviral disease is increasing, new hantaviruses are discovered continually, and already known hantaviruses are expected to spread to new areas. Therefore, knowledge and monitoring of these viruses are imperative from a public health perspective.

In this thesis, the characterization of a local human Puumala (PUUV) virus isolate is described. Genetical and serological relationships to other hantaviruses are investigated and the viral protein interactions, critical for genome packaging and assembly, are studied. We found that the nucleotide and amino acid sequences of the local PUUV strains are significantly different from the PUUV prototype strain Sotkamo, a difference that indicates that there might be a risk of misdiagnosing PUUV infected patients when using reagents derived from the prototype strain. These data contributed to the introduction of locally derived diagnostic tools to the Laboratory of Clinical Virology at the Umeå University hospital, which is the reference centre for hantaviral diseases in Sweden. Furthermore, when studying the underlying mechanisms of genome packaging, we identified several regions and amino acids absolutely required for nucleocapsid protein interactions. Also, a region that appeared to regulate this interaction was discovered. Finally, the serological immune responses in DNA-vaccinated mice and PUUV infected patients were investigated. We found that the cross-reactive antibody response in vaccinated mice and in infected individuals was unique and independent of homologous titres. Furthermore, four immunodominant epitopes with specific cross-reactive characteristics were identified.

Our findings have highlighted the complexity of the serological immune responses to hantavirus infections, and they emphasize the importance of customizing the diagnostic tools and performing clinical analyses on locally derived strains. In conclusion, we believe that these results are valuable in the development of new serological, genetic, and epidemiological tools.
List of publications

This thesis is based on the following original papers that are referred to by their Roman numerals.


### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>ANDV</td>
<td>Andes virus</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BCCV</td>
<td>Black Creek Canal virus</td>
</tr>
<tr>
<td>COS-1</td>
<td>Cell line derived from kidney cells of the African green monkey.</td>
</tr>
<tr>
<td>cRNA</td>
<td>Complementary RNA</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T-lymphocyte</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOBV</td>
<td>Dobrava virus</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>G&lt;sub&gt;n&lt;/sub&gt;/G&lt;sub&gt;c&lt;/sub&gt;</td>
<td>Glycoprotein located in the N-/C-terminus of the GPC</td>
</tr>
<tr>
<td>GPC</td>
<td>Glycoprotein precursor</td>
</tr>
<tr>
<td>GP</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>G-protein</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>HCPS</td>
<td>Hantavirus cardiopulmonary syndrome</td>
</tr>
<tr>
<td>HFRS</td>
<td>Hemorrhagic fever with renal syndrome</td>
</tr>
<tr>
<td>HTNV</td>
<td>Hantaan virus</td>
</tr>
<tr>
<td>L, M, S</td>
<td>Large, Medium and Small hantavirus gene segments</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>N</td>
<td>Nucleocapsid</td>
</tr>
<tr>
<td>NCR</td>
<td>Non coding region</td>
</tr>
<tr>
<td>NE</td>
<td><em>Nephropathia epidemica</em></td>
</tr>
<tr>
<td>NP</td>
<td>Nucleocapsid protein</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PUUV</td>
<td>Puumala virus</td>
</tr>
<tr>
<td>QPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>RdRp</td>
<td>RNA dependent RNA polymerase</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase-PCR</td>
</tr>
<tr>
<td>SAAV</td>
<td>Saaremaa virus</td>
</tr>
<tr>
<td>SEOV</td>
<td>Seoul virus</td>
</tr>
<tr>
<td>SNV</td>
<td>Sin Nombre virus</td>
</tr>
<tr>
<td>T&lt;sub&gt;c&lt;/sub&gt;-cell</td>
<td>Cytotoxic T-lymphocyte</td>
</tr>
<tr>
<td>T&lt;sub&gt;h&lt;/sub&gt;-cell</td>
<td>T-helper cell</td>
</tr>
<tr>
<td>TPMV</td>
<td>Thottapalayam virus</td>
</tr>
<tr>
<td>TSWV</td>
<td>Tomato spotted Wilt virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>TULV</td>
<td>Tula virus</td>
</tr>
<tr>
<td>vRNA</td>
<td>Viral RNA</td>
</tr>
<tr>
<td>wt</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
# Table of contents

1 INTRODUCTION ................................................................................. 1  
1.1 BRIEF HISTORY OF HANTAVIRUSES............................................. 1  
1.2 HANTAVIRAL DISEASES ................................................................. 3  
  1.2.1 Treatment ............................................................................... 5  
1.3 HANTAVIRUSES AND THEIR HOSTS ................................................ 5  
1.4 TRANSMISSION TO HUMANS ......................................................... 9  
1.5 HANTAVIRUSES IN SWEDEN ......................................................... 10  
1.6 THE VIRION ............................................................................. 11  
1.7 THE RNA POLYMERASE .............................................................. 12  
1.8 THE GLYCOPROTEINS ................................................................. 13  
  1.8.1 Glycosylation ......................................................................... 15  
1.9 THE NUCLEOCAPSID PROTEIN ................................................... 16  
  1.9.1 B-cell epitopes on the Nucleocapsid Protein ............................. 17  
1.10 THE HANTAVIRAL REPLICATION CYCLE ................................... 17  
  1.10.1 Virus entry ........................................................................... 17  
  1.10.2 Transcription and replication ............................................. 18  
  1.10.3 Translation, virion assembly, and release ............................ 20  
1.11 IMMUNOLOGY .......................................................................... 21  
  1.11.1 General concepts .............................................................. 22  
  1.11.2 Humoral immune responses to hantaviral infections .......... 23  
  1.11.3 Cross-reactivity ................................................................. 25  
  1.11.4 Cellular immune responses to hantaviral infections ........... 26  
1.12 HANTAVIRAL DIAGNOSTICS ..................................................... 27  
  1.12.1 Immunofluorescence analyses (IFAs) .................................. 27  
  1.12.2 Enzyme Immunoassays (EIA) .............................................. 27  
  1.12.3 PRNTs and FRNTs ............................................................. 28  
  1.12.4 Reverse-transcription PCR (RT-PCR) ................................. 29  
  1.12.5 Virus isolation ................................................................. 29  
  1.12.6 Serological rapid-tests ....................................................... 29  
1.13 VACCINES ................................................................................. 30  
  1.13.1 Brief history of vaccination ............................................... 30  
  1.13.2 Vaccines against hantaviruses ......................................... 31  
  1.13.3 DNA-vaccination ............................................................. 32  
  1.13.4 Animal models for hantavirus infections ............................ 36  
2 AIMS ............................................................................................. 38  
3 RESULTS AND DISCUSSION ........................................................... 39  
  PAPER I: CHARACTERIZATION OF A HUMAN Puumalavirus ............ 39  
  PAPER II: NUCLEOCAPSID PROTEIN INTERACTIONS .................. 41  
  PAPER III: DNA-VACCINATION OF BALB/C MICE ....................... 44
INTRODUCTION

1 INTRODUCTION

1.1 Brief history of hantaviruses

Viruses of the *hantavirus* genus, within the *Bunyaviridae* family, cause two diseases in man: Hemorrhagic fever with renal syndrome (HFRS) and Hantavirus cardiopulmonary syndrome (HCPS).

Records of outbreaks of hantaviral-like diseases in China date as far back as 960 A.D. By the beginning of the 20th century, several diseases were documented that were later ascribed as hantaviral infections (Lee, 1996). Illnesses resembling Hemorrhagic Fever with Renal Syndrome were recorded in Russia in 1913 (Casals et al., 1970). During the first World War, “War nephritis” was a major problem among British troops (Lee, 1996). In the 1930s two Swedish physicians described, independently of each other, a disease that later became known as *Nephropathia epidemica* (NE), a disease that displayed the characteristics of HFRS, albeit in a milder form (Myhrman, 1934; Zetterholm, 1934). During the second World War, Japanese, Soviet, and German troops suffered severe illnesses that were later deemed to be hantavirus infections (Lee, 1996). However, not until the Korean War (1950-1953), were hantaviral diseases really brought to the attention of western medicine. During this war, thousands of UN soldiers stationed in Korea fell ill and sometimes died in a disease soon named Korean hemorrhagic fever (later more generally known as HFRS). This outbreak became the starting point of an intense search for the etiological agent (Lee, 1996). More than twenty years later, in 1976, Ho Wang Lee and co-workers managed to detect viral antigens in the lungs of the black-striped field mouse (*Apodemus agrarius*) by immunostaining (Lee and Lee, 1976). Two years after that initial finding
was the causative virus isolated from an *Apodemus agrarius*, captured near the Hantaan river in Korea (Lee et al., 1978). This newly discovered Hantaan virus (HTNV) became the prototype hantavirus, a genus that would soon include many more virus serotypes. In 1979, soon after the isolation of the Hantaan virus, the connection of NE to HFRS was confirmed (Lee et al., 1979; Svedmyr et al., 1979). And in 1980, the causative agent of NE was identified in the lungs of bank voles (*Myodes glareolus*) captured near the Finnish village of Puumala and thus this new virus was named Puumala virus (PUUV) (Brummer-Korvenkontio et al., 1980). A few years later, Lee and co-workers isolated yet another hantavirus from wild urban rats, a virus later known as the Seoul virus (SEOV), named after the capital city of South Korea (Lee et al., 1982). In 1992, the Dobrava virus (DOBV) was isolated in Yugoslavia (Avsic-Zupanc et al., 1992), and a year later the first American hantavirus highly pathogenic to humans – the Sin Nombre virus (SNV) – was identified in the Four Corners region of USA (Nichol et al., 1993). Many more apathogenic and human pathogenic hantaviruses have been discovered in the Americas since then, the most noteworthy being the Andes virus (ANDV), the only hantavirus for which human-to-human transmission has been reported (Enría et al., 1996; Ferres et al., 2007; Martinez et al., 2005). In 1999, the Saaremaa virus (SAAV) was discovered in Estonia (Nemirov et al., 1999). This virus was first categorized as a Dobrava virus (Dobrava-Aa), but the debate is ongoing as to whether SAAV is a variant of the Dobrava strain or a new hantavirus species (Klempa et al., 2003; Klempa et al., 2005; Plyusnin et al., 2003; Plyusnin et al., 2006; Sironen et al., 2005).

Interestingly, the first hantavirus that was discovered was actually the Thottapalayam virus (TPMV), isolated from a shrew in India in 1964.
INTRODUCTION

(Carey et al., 1971). However, at the time the TPMV could not be correctly classified. Thus, only when the Thottapalayam virus later was re-discovered could this early finding be properly recognised (Carey et al., 1971; Tang et al., 1985; Zeller et al., 1989).

1.2 Hantaviral diseases

HFRS – Hemorrhagic fever with renal syndrome – is mainly a Eurasian disease caused by several different hantaviruses. Typical examples are Hantaan virus, Seoul virus, Puumala virus, Dobrava virus and Saaremaa virus. These Old World viruses cause infections of varying severity from asymptomatic infections and mild influenza-like symptoms to full-blown systemic infections with mortality rates of up to 15%. HCPS – Hantavirus cardiopulmonary syndrome – is found exclusively in the Americas. A large number of these New World HCPS-causing hantaviruses have been identified, but the most well-known are the Sin Nombre virus and the Andes virus. These viruses are prevalent in North- and South America, respectively, and cause very severe infections. The Sin Nombre virus has an estimated fatality rate of 40%, and the Andes virus can display a case fatality of up to 50% due to the sometimes lacking medical care in rural South America (Castillo et al., 2001; Hooper et al., 2001b; Schmaljohn, 2001).

HFRS has five more or less distinguishable phases with an abrupt onset of the disease. There is a febrile, flu-like, phase often accompanied by headache, abdominal pain, backache and nausea. Blurred vision is sometimes reported to occur and by the end of this phase hemorrhagic manifestations can be observed. The second phase is characterized by
hypotension. During this stage, there is proteinuria and a decreased level of platelets in the blood. About one-third of the deaths occur during this phase due to irreversible shock. The third phase is the oliguric phase where urine production decreases significantly due to the pronounced renal involvement of the disease, and blood creatinine often rises. The fourth phase is the polyuric stage which usually is an indication for recovery and the fifth and final convalescent phase (Krüger, 2001; Settergren, 1988).

An HCPS-infection starts with a prodromal phase with fever, nausea, headaches and myalgia. This phase is followed by a cardiopulmonary phase characterized by cough and shortness of breath which rapidly progresses and necessitates hospitalization and usually also mechanical ventilation. Intestinal and pulmonary oedemas are observed as well as hypotension and tachycardia. Cardiovascular collapse and shock at this stage of the disease is often the cause of death. This phase is followed by a diuretic phase which is characterized by rapid clearance of oedemas and the resolution of fever and shock. The following convalescent phase usually leads to full recovery (Schmaljohn, 2001; Nichol, 1996).

Apart from the obvious difference in severity, the principal distinction between HFRS and HCPS are the organs affected by the infection. For HFRS, renal complications are characteristic and these patients may require dialysis. In HCPS, an acute effect on lung function is frequently observed, necessitating mechanical ventilation. However, these two diseases also have many similarities. They are both febrile illnesses with acute onset and hemorrhagic manifestations. Furthermore, the renal involvement of typical HFRS is also evident in HCPS patients albeit in a
much lesser degree, and HFRS patients may have compromised lung-function as observed in typical HCPS. The similarities of these diseases, however, should not be surprising considering the close genetic relationship between the hantaviruses causing HFRS and HCPS, respectively (Khaiboullina et al., 2005; Krüger et al., 2001; Linderholm and Elgh, 2001; Nichol et al., 1996; Schmaljohn, 2001).

1.2.1 Treatment

Currently no treatment can cure hantaviral infections. Instead, the care of hantavirus-infected patients relies on supportive care and symptomatic treatment. Ribavirin, an antiviral drug, has been reported to reduce mortality in HFRS-patients, but no such effect could be determined from Ribavirin treatment of HCPS patients (Huggins et al., 1991; Mertz et al., 2004). However, presently Ribavirin is not approved by the U.S. Food and Drug Administration or the World Health Organization for treatment of hantaviral infections (Maes et al., 2004). Consequently, treatment of hantavirus infections, especially in endemic regions, need to be developed, and the search for more effective antiviral agents and prophylactic treatments is ongoing.

1.3 Hantaviruses and their hosts

The hantaviruses are maintained in rodents generally assumed to be persistently infected. Despite the severe hemorrhagic fevers observed in man, the rodents appear relatively unaffected by these infections (Meyer and Schmaljohn, 2000). So far, almost all of the known hantaviruses have been isolated from different rodent species. The exception is the Thottapalayam virus, which was isolated from an insectivore host the
Asian Musk shrew. However, increasing molecular evidence indicates the presence of hantaviruses in other insectivores as well (Arai et al., 2008; Arai et al., 2007; Klempa et al., 2006; Song et al., 2007), but these viruses remain to be isolated. Thus, in this thesis, the hantaviruses will be described as being rodent-borne.

At the time of writing this thesis, there were 22 serologically and genetically distinct hantavirus sero-types (as defined by the International Committee on Taxonomy of Viruses (ICTV)) (Table 1). However, recent discoveries of multiple new hantavirus strains have complicated the previously distinct hantavirus serotype definitions. Thus, as new hantavirus strains are found – possibly filling in the gaps between defined hantavirus species – the established hantaviral classification might need to be revised. With current definitions, each of the identified sero-types is maintained in one principal rodent host, although a few of the hantaviruses have been found in multiple host species. This host preference of the hantaviruses limits their geographical distribution to their host habitats. Because the different virus species cause diseases of varying severity, the clinical picture of hantavirus infections is different depending on location. Generally, hantaviruses carried by Murinae rodents are found in Euraisa and causes HFRS. Most cases are reported in China, and these Asian infections are more often quite severe (Krüger et al., 2001) (Fig. 1). In contrast, the hantaviruses carried by Arvicolinae rodents cause NE, the milder form of HFRS (Kallio-Kokko et al., 2005). These viruses are predominantly found in Europe and Russia, and the non-pathogenic ones have also been found in North America (Schmaljohn and Hjelle, 1997). Finally, hantaviruses carried by Sigmodontinae rodents.
## Table 1. Hantaviruses and their hosts according to the International Committee on Taxonomy of Viruses (ICTV).

* According to (Schmaljohn and Hjelle, 1997).

<table>
<thead>
<tr>
<th>Classification of host</th>
<th>Species</th>
<th>Abbreviated name</th>
<th>Disease*</th>
<th>Reservoir</th>
<th>Distribution of virus*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Order Rodentia, family Muridae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Subfamily Murinae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amur**</td>
<td>AMRV</td>
<td>HFRS</td>
<td>Apodemus peninsulae</td>
<td>Far east</td>
<td></td>
</tr>
<tr>
<td>Dobrava-Belgrade</td>
<td>DOBV</td>
<td>HFRS</td>
<td>Apodemus flavicollis</td>
<td>Central Europe, Balkans</td>
<td></td>
</tr>
<tr>
<td>Hantaan</td>
<td>THNV</td>
<td>HFRS</td>
<td>Apodemus agrarius</td>
<td>China, Russia, Korea</td>
<td>Nilvinter confucianus</td>
</tr>
<tr>
<td>Saaremaa**</td>
<td>SAAV</td>
<td>HFRS</td>
<td>Apodemus agrarius</td>
<td>Europe</td>
<td></td>
</tr>
<tr>
<td>Seoul</td>
<td>SEOV</td>
<td>HFRS</td>
<td>Rattus norvegicus</td>
<td>Worldwide, predominantly Asia</td>
<td>Rattus rattus, Rattus losea</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Subfamily Arvicolinae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isla Vista</td>
<td>ISLAV</td>
<td>nd</td>
<td>Microtus californicus</td>
<td>USA</td>
<td></td>
</tr>
<tr>
<td>Puumala</td>
<td>PUUV</td>
<td>HFRS</td>
<td>Myodes glareolus, Myodes rufocanus, Eothenomys regulus</td>
<td>Europe, Russia</td>
<td></td>
</tr>
<tr>
<td>Prospect Hill</td>
<td>PHV</td>
<td>nd</td>
<td>Microtus ochrogaster, Microtus pennsylvanicus</td>
<td>North America</td>
<td></td>
</tr>
<tr>
<td>Topografov</td>
<td>TOPV</td>
<td>nd</td>
<td>Lemmusibiricus</td>
<td>Siberia</td>
<td></td>
</tr>
<tr>
<td>Tula</td>
<td>TULV</td>
<td>nd</td>
<td>Microtus arvalis, Microtus ossaemomensialis</td>
<td>Europe</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Subfamily Sigmodontinae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Andes</td>
<td>ANDV</td>
<td>HCPS</td>
<td>Oligoryzomys longicaudatus, Oligoryzomys chacoensis, Oligoryzomys flavescens</td>
<td>Argentina, Argentina, Bolivia</td>
<td></td>
</tr>
<tr>
<td>Black Creek Canal</td>
<td>BAVV</td>
<td>HCPS</td>
<td>Oryzomys palustris</td>
<td>Argentina</td>
<td></td>
</tr>
<tr>
<td>Bayou</td>
<td>BCCV</td>
<td>HCPS</td>
<td>Sigmodon hispidus</td>
<td>USA</td>
<td></td>
</tr>
<tr>
<td>Cano Delgado El Morey Canyon</td>
<td>CADV</td>
<td>nd</td>
<td>Sigmodon albionis</td>
<td>South America</td>
<td></td>
</tr>
<tr>
<td>Khabarrovsk</td>
<td>KBRV</td>
<td>nd</td>
<td>Moropus fortis</td>
<td>Russia</td>
<td></td>
</tr>
<tr>
<td>Laguna Negra</td>
<td>LANV</td>
<td>HCPS</td>
<td>Calomys laucha</td>
<td>South America</td>
<td></td>
</tr>
<tr>
<td>Muleshoe</td>
<td>MULV</td>
<td>nd</td>
<td>Sigmodon hispidus</td>
<td>USA</td>
<td></td>
</tr>
<tr>
<td>New York</td>
<td>NIVY</td>
<td>HCPS</td>
<td>Peromyscus leucopus</td>
<td>USA</td>
<td></td>
</tr>
<tr>
<td>Rio Mamone</td>
<td>RIOMV</td>
<td>nd</td>
<td>Oligoryzomys microps</td>
<td>Bolivia</td>
<td></td>
</tr>
<tr>
<td>Rio Segundo</td>
<td>RIOSV</td>
<td>nd</td>
<td>Reithrodontomys mexicanus</td>
<td>Costa Rica</td>
<td></td>
</tr>
<tr>
<td>Sin Nombre</td>
<td>SNV</td>
<td>HCPS</td>
<td>Peromyscus leucopus, Peromyscus maniculatus</td>
<td>USA, Canada, Mexico</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Order Insectivora, family Soricidae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thottapallyam</td>
<td>TPMV</td>
<td>nd</td>
<td>Suncus murinus</td>
<td>India</td>
<td></td>
</tr>
</tbody>
</table>

** Not officially recognised by the ICTV.
nd – non documented
cause severe HCPS and are found only in North and South America. The Seoul virus is an exception to this generalized hantavirus distribution. SEOV is carried by the black rat (*rattus rattus*) or the brown rat (*rattus norvegicus*). As these rat species are found throughout the world, the SEOV may consequently be found in many locations (Glass et al., 1994; Heyman et al., 2004; Iversson et al., 1994; Schmaljohn and Hjelle, 1997).

To add further to the complexity of hantaviral distribution, there are areas where different rodent species co-exist, and thus the co-existence of several hantavirus sero-types is possible. For example, DOBV, PUUV and SAAV all occur in central Europe (Kallio-Kokko et al., 2005), and HTNV, SEOV and PUUV coexist in Asia (Li, 2007). In Russia, as many as eight hantavirus species have been identified (Tkachenko et al., 2007) and in the Americas there are twelve distinct hantavirus species defined to this day (ICTV).

![Figure 1. Phylogenetic tree of hantavirus genera (I).](image)

1.4 Transmission to humans

Hantaviruses are transmitted to humans through inhalation of contaminated excreta (such as urine, saliva, and faeces) or by direct physical contact with infected animals (Lee, 1996). Considering the route of infection in combination with knowledge of behavioural patterns of the rodents, certain activities can be defined as risk-behaviour: e.g., cleaning sheds or previously vacant summer houses where infected rodents are found and handling firewood from woodpiles or woodsheds. Occupational risk groups include forestry workers and farmers. In short, risk activities are those which may raise dust containing hantavirus particles. The route of virus transmission through inhalation holds true for all hantaviruses, and generally a human host is a dead-end for the virus since person-to-person transmission generally does not occur. However, in the case of Andes virus infections, person-to-person transmission has been described (Ferres et al., 2007; Martinez et al., 2005). Although this person-to-person transmission seems to be very rare, it is still a considerable public health issue considering the severity of Andes virus infections. Still, human-to-human transmission for other hantaviruses should not be dismissed too easily. The occurrence of family clusters of NE-patients is not uncommon, and hantaviral RNA has been demonstrated in the saliva of PUUV infected individuals (Pettersson et al., 2008).
INTRODUCTION

1.5 Hantaviruses in Sweden

In Sweden, the only hantavirus found thus far is the Puumala virus (PUUV), which causes *Nephropathia epidemica*, a mild form of HFRS. NE is common in northern Sweden and it is a very serious viral infection as the fatality rate is approximately 0.1-1% (Kallio-Kokko et al., 2005; Khaiboullina et al., 2005). There is an average of 200-500 diagnosed cases each year, but up to 1000 cases and above during the years when the bank voles (*Myodes glareolus*) (Fig. 2) – the rodent hosts of PUUV – are particularly abundant. During 2007, e.g., which was a peak year vole population-wise, there were 2195 NE cases reported in Sweden (Swedish Institute for Infectious Disease Control, SMI). The same year, a similar
outbreak was reported in Germany (Hofmann et al., 2008), indicating large vole populations in central Europe as well. In 2007, 1964 (i.e., ~90%) of the Swedish cases occurred in four of the northern counties: Västerbotten, Norrbotten, Västernorrland, and Jämtland. The true number of infected individuals, however, is probably 7-8 times higher as determined by seroprevalence studies in PUUV endemic regions (Ahlm et al., 1994).

1.6 The Virion

Viruses belonging to the hantavirus genus are negative stranded RNA viruses. The virions are spherical with an average diameter of 100 nm (80-120) although variable forms have been reported (Fig. 3 & 4). The virus particles consist of a lipid bilayer interspersed with two glycoproteins, denoted Gα and Gc (formerly known as G1 and G2), in a grid-like pattern. These glycoproteins form heterodimers that are visible by electron microscopy as spike-like projections of approximately 6 nm. Inside the virion, there are assumably equimolar amounts of the tripartite genome and each gene segment is denoted by its size: the large (L) segment (~6.5 kb) encodes an RNA dependent RNA-polymerase (RdRp); the medium (M) segment (~3.7 kb) encodes the glycoprotein precursor (GPC) which is cleaved into the two glycoproteins; and the small (S) segment (~1.8 kb) encodes the nucleocapsid protein (NP). The RNA segments are complexed to nucleocapsid proteins forming three stable ribonucleocapsid structures – ribonucleoproteins (RNPs) – within the virus particle (Elliott, 1996; Khaiboullina et al., 2005) (Fig. 3).
INTRODUCTION

Nucleocapsid protein (NP)
Amino-terminal glycoprotein (Gn)
Carboxy-terminal glycoprotein (Gc)
RNA dependent RNA polymerase (RdRp)
Nucleocapsid trimer encapsidating the negative stranded RNA

Figure 3. Schematic picture of a hantavirus particle.

1.7 The RNA polymerase

The RNA-dependent RNA polymerase (RdRp) (~240 kDa), the most conserved of the hantavirus proteins, has a central role in the virus replication cycle. The RdRp must perform a number of enzymatic functions such as endonuclease, transcriptase, replicase, and perhaps also helicase activities. The mutational frequencies of hantavirus RdRps is approximately $1 \times 10^{-3}$ subs/site/year, indicating that the RdRp does not have proofreading ability (Elliott, 1996; Ramsden et al., 2008; Schmaljohn, 2001). However, the true mutational rate of the virus is lower, as the majority of mutations will not outmatch the already established genotype (Sironen et al., 2001).
1.8 The glycoproteins

When glycosylated, the $G_c$- and $G_t$ glycoproteins are 72-74 kDa and 55-57 kDa in size, respectively, and rich in cysteine residues. Although the glycoproteins (GPs) are the least conserved of the hantaviral proteins (34% and 44% amino acid identity respectively), the positions of the cysteine residues and, to some extent, the sugar residues are extremely...
INTRODUCTION

conserved which indicates that the overall structures of the GPs in fact are similar (Elliott, 1996; Schmaljohn, 2001; Sheshberadaran et al., 1988). The GPs are type I membrane-spanning glycoproteins and, as such, span the lipid bilayer only once. In the virus infected cell, the amino-terminus of the glycoproteins are oriented inward, towards the ER-lumen, where they are glycosylated (Elliott, 1996; Khaiboullina et al., 2005).

The function of the glycoproteins at the surface of the virus particle is to mediate cell adhesion, attachment, and fusion. The virus entry into cells has been demonstrated to occur via integrin receptors. The β3-integrins mediate cell entry of human pathogenic hantaviruses and β1-integrins mediate the cell entry of the non-human pathogenic hantaviruses (Gavriloiskaya et al., 1999; Khaiboullina et al., 2005). Another important function of the G-proteins in viral infectivity has been proposed to entail the escape of the virion from the lysosome. This has been demonstrated for the La Crosse virus (member of the Orthobunyavirus genus, the Bunyaviridae family), where the Gn protein will undergo conformational changes in response to a lowered pH (Pekosz and González-Scarano, 1996).

Other proposed roles of the glycoproteins concern their C-terminal ends. Gn has a long cytoplasmic tail of ~150 aa, and Gc has a shorter tail, less than 9 aa. The long tail of Gn has been proposed to act as a substitute for a matrix-protein interacting with the nucleocapsid protein. This interaction may occur through the YRTL motif of the Gn-tail as this motif has earlier been shown to be implicated in intracellular signalling (Schmaljohn, 2001).
INTRODUCTION

1.8.1 Glycosylation

Glycosylation of a protein, the process of addition of sugars in the ER and the subsequent trimming of said sugars in the ER and Golgi compartments, is of the utmost importance when it comes to the production of biologically active proteins. The sugar residues are involved in numerous cellular activities such as folding, targeting, transport of proteins, modification of protein activity, recognition events, growth control, signal transduction and protection from proteolytic cleavage (Gabius et al., 2002; Hansen et al., 1997; Opdenakker et al., 1993).

Generally, glycosylation is either one of two types. There is N-linked glycosylation where a polysaccharide is co-translationally transferred and linked to the asparagine of a particular sequon (Asn-X-Ser/Thr or Asn-X-Cys where X can be any amino acid except proline). The other type of glycosylation, O-linked glycosylation, is when the sugars are added in the cis-Golgi compartment to a serine or threonine residue. For O-linked glycosylation, however, no clear consensus sequences have been identified as is the case for N-linked glycosylation (Hansen et al., 1997; Helenius and Aebi, 2001).

Hantavirus glycoprotein glycosylation has not been thoroughly investigated. The in silico propositions of N-linked glycosylation sites appear to be conserved within the respective groups HTNV/DOBV/SEOV/THAIV and PUUV/PHV/SNV (Avsic-Zupanc et al., 1995; Elliott, 1996; Nemirov et al., 1999). Still, the in vitro glycosylation tests indicate a heterogeneity of the functional glycosylation sites of the hantavirus G-proteins (Nemirov et al., 2003; Shi and Elliott, 2004; I).
1.9 The nucleocapsid protein

The nucleocapsid proteins (NPs) of hantaviruses are approximately 50 kDa and are constituted by 429-433 amino acids. The NP is the most abundant viral protein in the virion and in virus infected cells, and it has been proposed to be involved in several cellular functions.

The NP protects the viral RNA by forming stable ribonucleocapsid structures together with the RNA. The RdRp is also believed to be complexed to these ribonucleocapsids, but the exact mechanisms of these interactions are still unknown (Elliott, 1996). Interaction between different NP monomers is considered a key event in the formation of the RNPs, and towards this end, the NP monomers form trimers as an intermediate conformation (Alfadhlí et al., 2001; Kaukinen et al., 2001).

The NP interaction with viral RNA may occur by a specific recognition of the noncoding regions of the gene-segments. Also, the NP has been shown to display a higher affinity for the vRNA than to other RNA-types (Mir and Panganiban, 2004; Severson et al., 1999). The close interaction of the NP and the RNA also indicates that this protein has regulatory functions: The NP is believed to facilitate the synthesis of different types of viral RNA as it must dissociate from the RNA for the polymerase to be able to synthesize a new strand. This activity is supported by the demonstration that the NP, along with the RdRp, are absolutely necessary to obtain transcriptase activity (Lopez et al., 1995). The NP may also be involved in the termination of transcription and in regulating the switch between transcription and replication (Elliott, 1996; Schmaljohn, 2001).
1.9.1 B-cell epitopes on the Nucleocapsid Protein

The NP is the major antigen during the early serological immune response (Vapalahti et al., 1995). Consequently, the NP is widely used as an antigen in clinical diagnostics. Many studies have mapped antigenic determinants of hantaviral NPs, and have identified the amino-terminus of several hantaviruses to contain immuno dominant B-cell epitopes (Gedvilaite et al., 2004; Gött et al., 1997; Kang et al., 2001; Lundkvist et al., 1996b; Yamada et al., 1995; III, IV). However, depending on study design, somewhat different results are obtained. For example, using overlapping peptides in the mapping will reveal more complex antigenicity patterns and other epitopes than the amino terminal ones (Lundkvist et al., 2002; Tischler et al., 2005; Vapalahti et al., 1995). Nevertheless, the antigenic and immunogenic character of the NP makes this protein a common diagnostic antigen, but it is also widely used in prophylactic trials and epidemiological investigations (Geldmacher et al., 2004; Petraityte et al., 2008; Sandmann et al., 2005; Schmidt et al., 2005; Ulrich et al., 1998).

1.10 The Hantaviral replication cycle

1.10.1 Virus entry

Viruses of the Bunyaviridae family attach to eukaryotic cells via an interaction between the viral glycoproteins and host cell receptors. For the human pathogenic hantaviruses, αβ3-integrins are known to be involved (Gavrilouskaya et al., 1999), and recently Decay-accelerating-factor (DAF) has been proposed as a co-factor for entry (Krautkrämer and Zeier, 2008). Once the virus has attached to the cell surface, the virions are
taken up by clathrin-dependent receptor-mediated endocytosis, and the three ribonucleocapsids are released into the cytoplasm through fusion of the endosome and virion membranes in a low-pH dependant manner (Jin et al., 2002) (Fig. 5).

**Figure 5.** Schematic picture of the hantaviral lifecycle.

### 1.10.2 Transcription and replication

As soon as the viral gene segments are released into the cytoplasm, primary transcription of the vRNA into mRNA starts (Fig. 5 & 6). The
primers needed for initiation of transcription are acquired through cap-snatching where the 5’-caps of cellular mRNAs in the cytoplasm are cleaved off by the RdRp (Rossier et al., 1986; Schmaljohn, 2001). The mechanism of initiation of RNA synthesis in hantaviruses is believed to be of a “prime-and-realign” type. This mechanism is supported by the discrepancies observed in sequence analyses of 5’ ends of hantavirus mRNAs, where it has been shown to be a heterogeneity in the length of the untranslated regions (Garcin et al., 1995; Hutchinson et al., 1996).

Figure 6. Hantavirus transcription and replication strategy.

A yet unidentified signal induces a switch, from transcription to replication. A likely mechanism behind the switch may be through the accumulation of the newly synthesized NPs, but this is yet to be established (Schmaljohn, 2001). In addition, simultaneous transcription
and replication of vRNA may occur, but that too remains to be determined (Elliott, 1996). There is secondary transcription from the newly synthesized vRNA. This late transcription (with subsequent translation and replication) occurs at the same time as the newly assembled virus particles are released from the cell (Hutchinson et al., 1996).

1.10.3 Translation, virion assembly, and release

The translation of the L- and S-segment mRNAs is thought to occur on free ribosomes in the cytoplasm. The M-segment mRNAs, on the other hand, are thought to be translated by ribosomes on the ER for cotranslational cleavage and primary glycosylation. The glycoproteins are believed to form heterodimers in the ER and by doing so a localisation signal is created enabling retention in the Golgi compartment, where the trimming of the glycans and addition of terminal residues takes place (Antic et al., 1992; Elliott, 1996). The exact details of assembly and release of hantavirus particles are largely speculative. The general opinion for a long time was that all hantavirus particles were created by the budding of ribonucleocapsids into the Golgi compartment through an unknown mechanism. The new virions would then accumulate in the Golgi to finally be released through vesicular transportation. This was also a way of classifying the Bunyaviridae viruses since a majority of other negative stranded RNA viruses bud from the plasma membrane (Elliott, 1996; Schmaljohn, 2001). This theory of Golgi-budding is supported by the fact that the glycoprotein heterodimers are retained and embedded in the Golgi membrane with their carboxy-termini oriented outward towards the cellular cytoplasm (Elliott, 1996). However, studies on the BCCV and the
SNV reveal budding from the plasma membrane (Goldsmith et al., 1995; Ravkov et al., 1997), a finding that indicates a difference in morphogenesis between New World and Old World hantaviruses. However, the differentiation between these two groups of hantaviruses is not as simple as that: trafficking studies have clearly demonstrated accumulation of nucleocapsid proteins and glycoproteins in the Golgi compartment for both New and Old World viruses. These findings indicate the need for further studies on the subject (Schmaljohn, 2001). As for now, however, the possibility of dual sites of maturation cannot be ruled out.

1.11 Immunology

The body is constantly under attack from bacteria, viruses, and other harmful substances, but, luckily for us, the immune system is extraordinarily adept at confronting these incessant threats to our health. This immunological defence can be divided into two principal parts: innate and acquired immunity. The innate immunity is the first line of defence against invading microorganisms, and it is characterized by being unspecific and lacking immunological memory. The acquired immunity, on the other hand, is specific and has immunological memory. This is the part that can be “taught”, the part that evolves and responds to vaccinations. The acquired immunity response is delayed compared to the innate response, but once induced it is very efficient at handling specific pathogens.
1.1.1 General concepts

The acquired immune response can be divided into two parts: the cellular immune response and the humoral immune response. T-lymphocytes (T-cells) are the major components of the cellular immune system and particularly important when it comes to the clearance of intracellular infections like viruses. The T-cells are generally one of two types: either CD4+ MHC II-restricted T helper (Th)-cells or CD8+ MHC I-restricted cytotoxic T (Tc)-cells. The Th-cells recognize antigens presented in complex with MHC II molecules, and as a response they secrete several types of cytokine effector molecules. The Tc-cells recognise antigens presented in complex with MHC I molecules and will, when receiving co-stimulatory signals from Th-cells, mature into cytotoxic T-lymphocytes (CTLs) and kill altered self-cells.

The humoral immune response comprises B-lymphocytes (B-cells). These are white blood cells that produce antibodies (immunoglobulins). Efficient priming of B-cells results in the formation of antibody-secreting plasma cells and memory B-cells. For this proliferation to occur, the membrane-bound antibodies of the naïve B-cell must recognise an antigen. The B-cell also needs co-stimulatory signals from Th-cells. The end product, the antibodies, can be one of several types, and the antibody class will dictate its role in the overall clearing of infection.

Antigen presentation is a key event in the initiation of an immune response of the acquired immunity type. Without the proper presentation of an antigen, in the context of MHC molecules, no memory response will be achieved. MHC I-molecules are present on all nucleated cells and will
generally present antigens that are processed by the endogenous processing pathway. That is, proteins that are synthesized within the cell, such as viral proteins. Since Tc-cells recognize antigens presented on MHC I molecules, the CTL response is pivotal in the clearance of virus-infected cells.

MHC II-molecules are found only on antigen presenting cells (APCs) and present antigens that are processed in the exogenous processing pathway. The APCs take up extracellular components by phagocytosis or endocytosis, which will be processed by the endocytic processing pathway. This type of antigen presentation is recognised by Th-cells which are needed for, e.g., B-cell maturation.

However, the antigen presentation is not as clear-cut as following either the endogenous or the endocytic processing pathway. There are no impermeable barriers between the two processing pathways, and infecting viruses and intracellular bacteria may have their own inherent means to escape and/or manipulate the cellular processing machinery.

1.11.2 Humoral immune responses to hantaviral infections

Hantaviruses induce a strong antibody response in infected individuals. This antibody response almost always serves as the basis for diagnosing the patient. Furthermore, the detection of some antibody classes can be done for many years after the infection has been cleared from the body, indicating a possibly lifelong acquired immunity (Lundkvist et al., 1993; Settergren et al., 1991).

During the acute phase of hantaviral infections, there are detectable levels of the Immunoglobulin M (IgM) antibody against all three viral antigens—the nucleocapsid protein and the two glycoproteins. This very early IgM
The IgG response towards hantavirus proteins is detectable at later times than the initial IgM or IgA responses. Still, detectable levels of IgG antibodies are found in most cases when a patient is admitted. High neutralizing titres of IgG directed towards the \( G_n \) and the \( G_c \) proteins are observed and can be detected many years later (Bharadwaj et al., 2000; Valdivieso et al., 2006; Ye et al., 2004).

There are different opinions on the exact kinetics of the Immunoglobulin G response, however. According to some studies, IgG towards the NP comes first, whereas IgG antibodies directed towards the \( G_n \) and the \( G_c \) appear later (Kallio-Kokko et al., 2001). In HCPS-patients, however, the first sample always contains detectable levels of anti-NP and sometimes anti-\( G_n \) IgG (Bharadwaj et al., 2000; Borges et al., 2006; Tischler et al., 2005). Some studies indicate that the IgG response towards the \( G_n \) protein might arise before the anti-NP response (Elgh et al., 1995; Groen et al., 1992). Whether the IgG titres toward the NP rise before, simultaneously, or after the anti-\( G_n \) IgG titres is a matter of debate, but it is clear that the
anti-NP IgG titres initially increase faster than the anti-glycoprotein responses (Lundkvist et al., 1993).

Presence of specific IgE has also been demonstrated during the acute phase in HFRS-patients (Alexeyev et al., 1994), but the implications of this finding needs further investigation.

It is difficult to determine the kinetics of the antibody response in hantavirus infections, and the efforts are hampered by several factors: the uncertainty of the length of the incubation period (possibly in part due to dose of infecting virus), genetic background of the patients, patient admission at various stages of disease, and the sensitivity of different antibody detection techniques. Still, it is of great interest to physicians as well as researchers to understand the different aspects of the antibody response kinetics. The different antibody classes present in the serum of the patient may provide a good estimate on the elapsed time since initial infection, and the titre of neutralizing antibodies during the acute phase appears, in the case of HCPS-patients, to predict the severity of disease (Bharadwaj et al., 2000; Borges et al., 2006; Tischler et al., 2005).

1.1.1.3 Cross-reactivity

The antibody response of hantavirus infected individuals generally displays cross-reactive characteristics (Araki et al., 2001; Elgh et al., 1998; Elgh et al., 1997; Lundkvist et al., 1997; Sheshberadaran et al., 1988; IV) even though individualities have been found (IV). This cross-reactivity is particularly evident between viruses within the same rodent host group (Murinae-, Arvicolinae-, or Sigmodontinae rodents) (Elgh et al., 1997), and conclusive diagnoses can be difficult to make using enzyme immuno assays (EIAs) in areas where several hantavirus
serotypes co-circulate. Although the treatment of hantaviral infections is symptomatic, prompt and correct diagnoses are still desirable. Such early diagnoses could help predict the extent of supportive care required and would facilitate epidemiological surveys. There are data indicating extensive neutralizing cross-reactions in the acute and early convalescent phase, hampering early discrimination of the infecting serotype (Lundkvist et al., 1997), but other investigations in contrast indicate an increasing magnitude of cross-reactive antibody responses over time (Elgh et al., 1998). Either way, simple sero-typing would be beneficial.

1.11.4 Cellular immune responses to hantaviral infections

Hantaviruses induce a strong cellular immune response in infected individuals. The memory T-cell population increases shortly after the onset of disease along with an increase of other white blood cells and T-cell activation markers such as CD25, CD71 and HLA-DR (Maes et al., 2004). In addition, similar to the antibody response, the cellular immune response is long-lived and detectable for years after the initial infection (Van Epps et al., 2002). The CD8^+ T_c-cells are necessary for clearing a virus infection, but their inherent capacity to attack altered self-cells can, when unchecked, cause severe tissue damage (Kägi et al., 1994). Although the pathogenesis of hantaviral diseases is poorly understood, it is hypothesized to be caused mainly by the cellular immune responses (Temonen et al., 1996; Yanagihara and Silverman, 1990; Zaki et al., 1995).
1.12 Hantaviral diagnostics

There are several physiological characteristics of hantaviral infections. In the case of *Nepropathia epidemica*, early symptoms include fever, myalgia, headache, and blurred vision. Furthermore, there are basic clinical analyses that reveal thrombocytopenia, elevated levels of blood creatinine, hematuria and/or proteinuria (Settergren, 2000). Still, these symptoms are only indications of a hantaviral infection and the initial diagnosis must be confirmed by more specific testing.

1.12.1 Immunofluorescence analyses (IFAs)

The most widely used hantavirus specific clinical test today is the immunofluorescence analysis (IFA). IFA is based on serum immunoglobulin binding to virus-infected cells. Since hantaviruses were adapted to grow in cell culture in the 1980s, such infected cells are generally used for the IFAs as opposed to lung tissue from infected rodents, which results in more unspecific reactions. Generally, IFA is a reliable method assuming that the patient has developed an antibody response towards the virus. This is usually the case at the stage when a patient is admitted, but some individuals develop the antibody response late, several days after onset of symptoms (Kallio-Kokko et al., 1998). To properly diagnose these late responders, a second IFA analysis is needed on a serum sample drawn a few days later.

1.12.2 Enzyme Immunoassays (EIAs)

Similar methods, also based on antibody-detection, are the enzyme immunoassays (EIAs). But unlike the IFAs, which rely on propagation of virus, the EIAs are frequently based on recombinant antigens. These
INTRODUCTION

Antigens can be full-length proteins or truncated variants expressed either in bacteria (Elgh et al., 1997), insect cells (Vapalahti et al., 1996), or in yeast cells (Meisel et al., 2006; Razanskiene et al., 2004). The advantages of using these methods are their swiftness (diagnoses within hours), simplicity (enabling use in less equipped laboratories), and cost-effectiveness. The sensitivity of the EIAs is high and probably more reproducible than IFAs, but both methods might be needed for conclusive diagnoses (Kallio-Kokko et al., 1998).

1.12.3 PRNTs and FRNTs

Plaque reduction neutralization tests (PRNTs) or Focal reduction neutralization tests (FRNTs) is the gold standard when it comes to serotyping viruses. This is achieved by determining the neutralizing capacity of serum antibodies. In the case of the hantaviruses, the virus grows slowly in mammalian cells and displays a poor cytopathic effect, and the ability to form plaques decreases after too many passages in the frequently used VeroE6 cell line (Lee et al., 1999). In conclusion, there are difficulties with obtaining clear, good quality plaques. For this reason, the FRNT approach, which does not require cell-lysis, might be an alternative. The principle of the FRNT is to use a virus, of a previously determined titre, mixed with serially diluted patient serum samples. This mixture is later applied to susceptible cells and incubated to allow viable virus to infect. The virus-antibody complex is washed off and the cells are allowed to grow under an agar over-lyay, which hinders the newly produced virions to diffuse freely in the cell culture. The degree of virus neutralization is determined by visualizing the infected foci by staining with fluorescent or enzymatically labelled antibodies and counting the spots (Heider et al., 2001; Tanishita et al., 1984).
1.12.4 Reverse-transcription PCR (RT-PCR)

Another method to diagnose hantaviral infections is to demonstrate the presence of viral RNA by RT-PCR (Dekonenko et al., 1997; Hörling et al., 1995; Xiao et al., 1991). A further development of the PCR technique is to detect viral RNA in real-time (Q-PCR) and follow the viremia by quantification of viral RNA (Evander et al., 2007). This technique has been used to determine and monitor the viral load in Nephropathia epidemica patients. The concurrent monitoring of hantaviral antibodies revealed that the viremia, in many cases, is detectable before the earliest IgM response. Typically, the viremia appeared first and then gradually disappeared as the IgM and the IgG responses increased. Generally, the RNA is no longer detectable after the first week (4-9 days) of infection (Evander et al., 2007).

1.12.5 Virus isolation

The classical way of demonstrating a viral infection is to grow and isolate the virus by passage in cell-culture. The isolation of a hantavirus from rodent lung tissue is time-consuming and difficult, yet the isolation from a human source is even more difficult. Therefore, the isolation approach as a means of routine diagnostics is not reasonable (Lee et al., 1999).

1.12.6 Serological rapid-tests

As the incidence of diagnosed hantavirus infections has increased over the last few years, the need for simple and reliable rapid-tests has emerged. To meet this demand, some point of care tests (POC-tests) have been developed based on the N antigen of Puumala, Dobrava, and Hantaan hantaviruses. Although single antigen-tests are more sensitive
INTRODUCTION

and slightly more specific than the combination tests, both variants offer a good alternative when field-diagnoses are made (Hujakka et al., 2003). In addition, the commercially available POC-PUU test (POC PUUMALA, Reagena Ltd., Toivala, Finland) is also useful in the diagnosis of other hantavirus infections due to the cross-reactive characteristics of the nucleocapsid proteins (Navarrete et al., 2007).

1.13 Vaccines

1.13.1 Brief history of vaccination

There are more than one thousand year-old Indian and Chinese documentations of deliberate efforts of protection against infectious disease by “vaccination”, but the effectiveness of these early attempts is questionable. An early form of vaccination which actually produced the wanted effect, i.e., immunity, is known as variolation. This technique involved rubbing of dried puss from smallpox pustules into a small scratch on the arm. Although effective, it was very dangerous by modern standards as the method was actually the deliberate infection of a healthy person with a live and virulent strain of smallpox virus. Approximately 2-3% of the treated individuals died of smallpox contracted from the variolation (Bazin, 2003; Plotkin and Orenstein, 2004), but in a world where smallpox was rampant, causing extensive morbidity and mortality, that risk was perhaps worth taking.

In 1796, Dr. Edward Jenner introduced the first safe vaccination. He put into practise to infect people with cowpox, an infection relatively harmless to humans, as means of protecting against the more virulent smallpox virus. This method, however, is not generally applicable for
most infectious diseases since there are no naturally occurring related etiological agents that are both harmless and confer protection. Louis Pasteur was the first person to develop modern vaccines, similar to the vaccines of today. He managed to ‘attenuate’ a virulent strain of the chicken cholera bacteria. This weakened strain protected against the more virulent strains. A few years later, Pasteur became the first person to develop an attenuated rabies vaccine that was successfully administered to a boy badly bitten by a rabid dog. This deliberate administration of an infectious agent to a human being – although already infected and thus facing certain death – caused an outrage, but did save the boy’s life. Further development and advances in vaccinology include the introduction of killed vaccines, toxoid vaccines, subunit vaccines, recombinant protein vaccines, and genetic vaccines (Bazin, 2003; Plotkin and Orenstein, 2004).

1.13.2 Vaccines against hantaviruses

Considering the widespread distribution of hantaviruses, the severity of the diseases, and the lack of effective treatments, a hantavirus vaccine would be a great benefit. However, there is only one commercially available vaccine against hantaviruses: the Hantavax™ vaccine based on the hantaan virus cultivated in suckling mouse brain and subsequently formalin inactivated. The efficacy of the Hantavax™ vaccine has been questioned as the induced titres of neutralizing antibodies are low, and frequent boosters are necessary. Nevertheless, there has been a 45% decrease in the incidence of Korean HFRS since the vaccine was introduced in 1990 (Hjelle, 2002). However, the way the Hantavax™ vaccine is produced – in the brains of mice – prevents this vaccine from being approved for human use in most countries (Cho and Howard, 1999;
Cell-culture derived vaccine candidates have been tested in China (Song et al., 1992), but the problems of low neutralizing titre and short lasting immune responses remain the same as for the Hantavax™ vaccine. Recombinant alternatives could be developed, however. Recombinantly expressed glycoproteins and nucleocapsid proteins, or the M and/or S genes, confer protection in animal trials (Bharadwaj et al., 2002; Bucht et al., 2001; Chu et al., 1995; Custer et al., 2003; Dargeviciute et al., 2002; Klingström et al., 2004; Maes et al., 2008; Maes et al., 2006). There are even reports on cross-protection between different hantavirus serotypes (de Carvalho Nicacio et al., 2002; Hooper et al., 2001a), but no such safe hantavirus vaccine is yet commercially available.

1.13.3 DNA-vaccination

When Wolff and colleagues (Wolff et al., 1990) first demonstrated that naked genetic material – DNA or RNA – are expressed when injected into an organ, the research on naked DNA delivery flourished. A few years later, when Ulmer and colleagues (Ulmer et al., 1993) demonstrated that cDNA-injected animals were protected against an influenza virus challenge, the research on DNA-vaccines, also known as genetic vaccines, boomed. Not surprisingly, the research has been intense as researchers have seen the possibility to construct new, safe, and effective vaccines against diseases where no traditional, safe vaccines can be produced. However, this new research field has proven to be more complex than first expected, and the DNA-vaccine methodology has dealt with several problems when it comes to translating the successes in animal models to human use. However, these problems are on the way of being solved: about 200 ongoing clinical trials and five licensed DNA-
vaccines (for use in pigs, dogs, horses, salmon and people) are indications of the possibilities that this technique provides (Beláková et al., 2007; Kjeken et al., 2006; Liu et al., 2006; Weiner, 2008).

The basic principle of DNA-vaccination is that the antigens are produced within the cells of the vaccinee. In this way, the antigen presentation after DNA-vaccination is similar to the antigen presentation during a natural viral infection. There are several advantages of DNA-vaccination compared to traditional vaccine formulations. The advantage of vaccine design enabled by recombinant DNA techniques is obvious. In addition, DNA-vaccines are able to induce CD8+ T-cell responses, which are difficult to induce with conventional protein-based vaccines. Other major benefits of DNA-vaccines are their low cost and easy large-scale production. DNA is also a stable structure and does not require cooling, a characteristic that simplifies storage and transportation. These qualities of DNA-based vaccines are of great value when it comes to vaccination schemes, particularly in developing countries.

The general structure of DNA-vaccines is based on bacterial plasmids. These vectors typically contain a eukaryotic promoter such as the one from the human cytomegalovirus (hCMV) or from the simian virus 40 (SV-40). Then there is the gene of choice (GOI) encoding the antigen, followed by a polyadenylation signal. The insertion of the GOI is enabled by a multiple cloning site, and the plasmid contains a prokaryotic origin of replication and an antibiotic resistance gene to enable growth and selection in bacterial cultures (Beláková et al., 2007; Rice et al., 2008).
Depending on the encoded antigen, DNA-vaccination can induce both a humoral and a cellular immune response, but the exact mechanisms of DNA-vaccine induced immune responses are not fully elucidated. The most frequently used DNA-delivery method is intramuscular injection (i.m.), but there are numerous other routes of administration: needle injection (intra dermal, intravenous, subcutaneous, epidermal, intra peritoneal etc), gene-gun delivery, needle-free jet injection (Biojector), viral carrier systems (viral vectors), and DNA-carrier complex systems, just to mention some (Beláková et al., 2007). Gene-gun administration, the delivery method used in Paper III, is a method where DNA is coated onto small gold particles that are shot into the cells of the skin by a compressed helium-driven gun. The dendritic cells (DCs) – more specifically the epidermal Langerhans’ cells – are believed to play a major role in the immune response after gene-gun vaccinations. The Langerhans’ cells, immature DCs present in the stratum spinosum of the epidermis, when encountering an antigen, will migrate to lymphatic organs and activate a T-cell response (Beláková et al., 2007; Rice et al., 2008; Tsen et al., 2007). The actual initiation of an immune response can occur through different mechanisms, and depends on the type of cells being transfected by the antigen encoding DNA. However, the major route of induction is believed to occur through directly transfected somatic cells (keratinocytes, myocytes etc.). These cells are thought to constitute the majority of transfected cells both in gene-gun administration and in intramuscular injection, and they will present the antigen in an MHC I setting, but that will not initiate the strong immune response actually observed. Instead, the immune responses when transfecting non-APCs are explained by ‘cross-presentation’ to professional antigen presenting cells. Cross-presentation (or cross-
priming) is an indirect transfer of endogenously produced antigens, possibly through apoptotic vesicles, to other cells. This mechanism is expected to induce an antibody response and a CD4+ T-cell response in addition to a CD8+ response (Bevan, 1976; Carbone and Bevan, 1990; den Haan and Bevan, 2001; Rice et al., 2008). The reason for the strong CTL response is explained by the discovery that engulfed phagosomal material can fuse with vesicles from the ER, a process that allows exogenous antigen access to the MHC I loading pathway (Guermonprez et al., 2003). This mechanism of cross-presentation is generally believed to explain the re-presentation of exogenous antigen in MHC I and MHC II settings (Beláková et al., 2007; Rice et al., 2008).

The DNA-vaccination methodology has encountered many bumps on the road. The main problem has been to translate the encouraging results from animal studies into equal successes in human subjects. The efficiency of the genetic vaccines was at first simply too low. As it turned out, the inefficiencies were due to low transfection frequencies (Rice et al., 2008). There are various strategies to increase the transfection efficiency, i.e., the penetration of the plasma membrane and entry into the nucleus of the target cell. Different strategies include in vivo electroporation, the use of minicircle DNA, different carrier systems that enable more cell specific targeting, and using various targeting signals fused to the gene of choice (Beláková et al., 2007; Johansson et al., 2002). DNA by itself is not antigenic, but the bacterially derived plasmids contain CpG dinucleotide motifs. Because these motifs are non-methylated DNA that occur less frequently in human DNA, they act as innate vaccine adjuvants (Higgins et al., 2007). But the inherent CpG-motifs might not be sufficient to boost the elicited immune response. To
address this deficiency various tactics have been employed. Co-
expression of cytokines, chemokines, enzymes, pathogen-like structures,
and growth factors have all been tested for the potential to increase the
number of DCs present at the site of antigen expression (Liu et al., 2006).

Some safety concerns regarding DNA-vaccinations have been expressed.
The risk of integration of the genetic vaccine into the host genome is one.
This event could activate oncogenes or inactivate oncogene suppressors,
but although integration has been demonstrated, the frequency of these
side effects is very low (Beláková et al., 2007; Kjeken et al., 2006). Other
fears include transferring antibiotic resistance genes to other bacteria.
This risk remains to be evaluated, but one way around the problem could
be the use of minigenes. In addition, other concerns include the initiation
of autoimmune diseases and the fear of inducing tolerance rather than
immunity. Under extreme conditions, autoimmunity has been induced in
animal models, but no such responses have been observed using
therapeutic doses. Tolerance, however, is a problem in very young and
very old animals (Beláková et al., 2007), so there are safety concerns that
need to be addressed. Nevertheless, DNA-vaccines have generally proved
to be safe. Something that, according to Rice and Ottensmeier, 2008, is
reflected in the relaxation of the requirements to assess autoimmunity,
integration, and persistence in clinical trials, both by the US Food and
Drug Administration and European authorities.

1.13.4 Animal models for hantavirus infections

To evaluate the efficacy of vaccine candidates, animal models are needed.
However, for a long time, no satisfactory animal model for HFRS or
HCPS existed. A bank vole model is available, and this animal model has
been used to study Puimalavirus infections (Lundkvist et al., 1996a), yet,
this model cannot serve as a disease model since the voles do not develop any symptoms of disease. To study a more human-like disease, a Cynomolgus macaque model has been developed (Groen et al., 1995; Klingström et al., 2002). For HCPS caused by the Andes virus, a lethal disease model in Syrian hamster was developed in 2001 (Hooper et al., 2001b).
2 AIMS

The objectives of my work were: to characterize a human Puumala virus isolate, PUUV Umeå/hu; to identify regions of importance for the PUUV Umeå/hu NP multimerization; to elucidate the immunological relationship between three related hantavirus NPs; and to use that information to develop diagnostic and epidemiologic tools.
3 RESULTS AND DISCUSSION

The results of each of my papers in this thesis are summarized in the following section. For more details concerning materials and methods used or for more extensive results and discussion, please see the respective articles.

**Paper I: Characterization of a human Puumalavirus**

To better understand the genetic relationships between different hantaviruses and to improve local diagnostics, we have studied a Swedish hantavirus isolate. We have sequenced the entire genome of a local human Puumalavirus (PUUV) – PUUV Umeå/hu – isolated from a man from a village outside of Umeå. This cDNA sequence represents the first complete sequence of a Puumala virus strain derived from a human source.

When comparing the nucleotide (nt) sequences of the PUUV Umeå/hu strain with the prototype strain of the Puumala virus serotype (the Finnish Sotkamo strain), an overall nucleotide (nt) sequence diversity of 19% was revealed. Despite the fact that this nt difference translates into a smaller aa disparity, it is still noteworthy in the light that the Sotkamo strain was, at the time, used extensively for PUUV diagnostics. In areas where other PUU viruses circulate, there could be a potential risk of misdiagnosing NE patients due to the lower reactivity to the Sotkamo antigens. The heterogeneity of the PUUV genus has been pointed out before, mostly for strains originating from very distant locations. However, these differences are not necessarily correlated to geographical distance. For instance, nt-
differences of 7% was recently reported for strains collected only 10 km apart (Johansson et al., 2008).

Furthermore, when constructing phylogenetic trees for the S and M segments using full-length sequences derived from bank voles, we made an interesting finding. As expected, the Umeå/hu strain clusters together with other PUUV strains from northern Sweden. For the M segment, the Hällnäs/Vr. and Umeå/hu strain are most related, whereas for the S segment, Hällnäs/Vr. is more related to the Vindeln strain. This observation could indicate a possible segment exchange between the Hällnäs/Vr. and the Umeå/hu M segments. The close functional relationship between the RdRp and the NP is generally believed to disqualify S segment exchanges. This is no absolute truth, however. There are reports on possible S segment exchanges as well, although, the indications of M segment exchanges appear to be more conclusive (Henderson et al., 1995; Li et al., 1995).

The glycoproteins were investigated in an effort to identify functional glycosylation sites, since unlike the general opinion, protein glycosylation patterns are not always well defined. Glycosylation prediction software was used and, in total, six specific asparagines and three threonines were substituted with serines or alanines, respectively. The resulting glycosylation mutants were compared with the corresponding wild type peptide by gel-shift analysis. In total, four N-linked (N142, N357, N409, and N937) and one O-linked (T985) functional glycosylation sites were found. Interestingly, only two days prior to our submission of this manuscript (I), a similar study on HTNV G-protein glycosylation was accepted for publication (Shi and Elliott, 2004). The data on the HTNV
RESULTS AND DISCUSSION

glycoproteins, albeit addressing only N-linked glycosylation, agreed with our results. The four glycosylated asparagines we identified in PUUV GPs were found to be conserved in the HNTV GPs. In addition, a fifth functional N-linked glycosylation site was identified in the HNTV G\textsubscript{n}. This is indicative of the heterogeneity of the glycosylation sites of the hantavirus NPs, also summarized by Elliott (1996). Interestingly the postulated glycosylation sites are more conserved within the two groups HTNV/DOBV(SEOV/THAIV and PUUV/PHV/SNV, suggesting a possible importance of the glycosylations for the serological recognition.

This paper shows that PUUV Umeå/hu differs significantly from the Sotkamo strain, the Puumala prototype virus. Our data contributed to the introduction of locally derived diagnostic tools to the Laboratory of Clinical Virology at the Umeå University hospital, which is the reference lab for hantaviral disease in Sweden. Furthermore, these results have highlighted the large variability within the Puumala virus serotype, a variability that underlines the importance of customizing the diagnostic tools and performing clinical analyses on locally derived strains.

**Paper II: Nucleocapsid protein interactions**

Correct packaging of the viral genome and assembly of virus particles in infected cells are dependent on interactions between viral proteins. To increase the understanding of these crucial processes of the viral life cycle, we have studied the interactions of PUUV Umeå/hu nucleocapsid protein (NP) subunits using a Yeast-2-Hybrid system.
The amino-terminus of the NP was initially investigated as similar studies of related hantaviruses, like Sin Nombre and Tula, have shown that the amino-terminus of the NPs is important for the protein-protein interactions (Alfadhli et al., 2001; Kaukinen et al., 2003). Studying the amino-terminus of the PUUV Umeå/hu reveals the presence of two putative coiled-coil domains that are common protein binding motifs (Lupas, 1996). Hence NP deletion mutants were constructed and assayed with respect to interaction with the full length NP. We found that the deletion of the coiled-coils, one at a time or simultaneously, increased the interaction as measured by expression of the lac Z reporter gene. Hence our results indicated that the coiled-coils (aa 1-99) are involved in the interaction and multimerization of the NPs, but they have a regulatory – inhibiting – function. Furthermore, a short region (aa 100-120) appears to be necessary to maintain the NP-NP interaction, as a deletion of this region lowered the interaction to one-third compared to full-length wt NP-NP interactions.

In comparable interaction studies performed on other NPs, the carboxy-terminus has been demonstrated to contain essential elements for protein-protein interactions. In the SNV, several regions of the carboxy terminus are absolute prerequisites for interaction (Alfadhli et al., 2001), and similar findings have been reported for the TULV (Kaukinen et al., 2003), the SEOV (Yoshimatsu et al., 2003), and for the Tomato Spotted Wilt Virus (TSWV) (Uhrig et al., 1999). Therefore, four postulated helix regions in the carboxy-terminus were investigated. The four helices were deleted, one at a time, and three of the helices, when removed, turned out to have a major effect on the NP-NP interaction. The interaction was practically abolished by the deletion of these helices. Furthermore, five
RESULTS AND DISCUSSION

aromatic amino acid residues were identified within these helices as being absolute requirements for the NP-NP interactions.

Thus the collective data on NP homotypic interactions all agree that regions of the carboxy-terminus are necessary even though these investigations differ in design and precision (Alfadhlî et al., 2001; Kaukinen et al., 2001; Kaukinen et al., 2003; Yoshimatsu et al., 2003). The data on the amino-terminal involvement in the NP-NP interactions is slightly more complex however as it is somewhat contradictory. Recent crystallization of amino-terminal fragments of the SNV NP revealed the formation of amino-terminal antiparallel coiled-coils but not any intermolecular interaction (Boudko et al., 2007). This may indicate that these amino-terminal coiled-coils are insufficient to initiate the trimerization of the full-length molecule. These findings are inconsistent with the proposed model of trimerization where the tips of the coiled-coils supposedly are the first to come into contact and trigger the intermolecular trimerization event (Alminaite et al., 2008). On the other hand, studies on the Marburg virus (Filoviridae family) RNP formation indicate the necessity of the coiled-coils. These investigations revealed that the coiled-coil motif, when fused to a reporter protein, was enough to mediate interaction with another NP amino-terminus (DiCarlo et al., 2007). Furthermore, our analyses of the PUUV NP amino-terminus, where the coiled-coils are not necessary for the homotypic NP interactions, are similar to the findings in SEOV, DOBV and to some extent HTNV (Yoshimatsu et al., 2003). In contrast, the importance of the coiled-coils has been demonstrated by interaction studies on TULV and SNV NPs (Alfadhlî et al., 2001; Kaukinen et al., 2003). This is despite that the two latter serotypes are genetically more similar to PUUV. In
conclusion, all studies implicate both amino- and carboxy-termini in the NP-NP interactions. However, the importance of the coiled-coils and whether the trimerization and subsequent RNP formation follows a head-to-tail or a head-to-head/tail-to-tail mechanism remains to be elucidated.

**Paper III: DNA-vaccination of Balb/c mice**

The serological cross-reactions between different hantaviruses are extensive, but not fully understood. To discern these serological relationships, we have analysed the antibody responses in mice after vaccination with three phylogenetically distinct and geographically separated hantavirus NP encoding genes: the S-genes of PUUV Umeå/hu, SEOV (Sapporo SR-11) and SNV (Convict Creek 107).

For evaluation of the antibody response, antibody titres were determined for each individual mouse. Serum titres towards the homologous antigens, as determined by ELISA and Western blot, were first determined as a means of assessing the DNA-vaccination procedures. All of the vaccinated mice exhibited high titres of anti-NP antibody compared to the negative controls. Furthermore, the titre levels within each group towards the homologous antigen were uniform, an indication of the success of the immunizations. However, we discovered that the cross-reactive antibody response for each individual mouse was unique with individual antibody responses ranging from no detectable cross-reactivity to cross-reactive titres comparable to the homologous titres. These discrepancies were investigated further by using NP deletion mutants and performing a crude epitope-mapping. We identified an amino-terminal region containing
immuno-dominant epitopes and in this region two epitopes were postulated.

Other investigations are in line with our findings. Epitope mappings of several different hantavirus NPs place the immunodominant epitopes in the amino-terminus (Elgh et al., 1996; Jenison et al., 1994; Lundkvist et al., 1996a; Lundkvist et al., 2002; Yamada et al., 1995; Yoshimatsu et al., 1996). The cross-reactivities of the hantavirus NPs, on the other hand, have not been as thoroughly investigated. One approach to eliminate cross-reactions and allow more precise serotyping has been to remove the entire NP amino-terminus or selected conserved amino-terminal regions (Araki et al., 2001; Wang et al., 1993). To remove large portions of the amino terminus of a hantavirus NP will eliminate many unwanted cross-reactions, but as many immunodominant epitopes are located within this region, the overall antibody reactivity will drop significantly.

The pronounced individual cross-reactivity that we observed was unexpected, particularly since the mice that we used were inbred and thus genetically identical. Yet, there were no external factors, such as infections of the mice, noticed that could explain such discrepancies. The answer might lie in how the antigenic determinants are presented to the immune system, but as the mice have the same MHC haplotypes, additional explanations are clearly needed. One aspect that could be part of the explanation is the fact that the cross-reactive epitopes (i.e., the conserved epitopes that are shared by several hantavirus NPs) are likely quite few in comparison to the total number of epitopes displayed on these proteins. We believe that the differences in the cross-reactivity that was observed in the DNA-vaccinated mice reflect this scarceness of
RESULTS AND DISCUSSION

common epitopes in an abundance of sero-specific antigenic determinants. The initial antigenic recognition of the very first immunization is likely only enhanced by the subsequent boosters, therefore, the idea of several individuals responding identically to a peptide literally littered with epitopes is perhaps not the most likely scenario. Still, the mice are supposedly genetically identical and were vaccinated with identical formulations. Perhaps the explanation to the differences could be of epigenetic origin. Data on identical twin pairs indicate the substantial influence of (generally) non inheritable genome modifications on gene expression and phenotype. Such differences are also manifested in inbred mice (Dolino et al., 2007; Fraga et al., 2005; Weaver et al., 2004; Whitelaw and Whitelaw, 2006; Wong et al., 2005). Nevertheless, regardless of the underlying mechanisms of the individualities of the vaccinated mice, the idea of identifying conserved, i.e., cross-reactive, epitopes is quite compelling. The removal of such antigenic determinants could generate serotype specific antigens that would greatly facilitate diagnostics and the serotyping of hantaviral infections.

We believe that our study’s description of individual variations and regions containing dominant antigenic determinants for homologous and heterologous (cross-reactive) antibody recognition could have diagnostic value. Further knowledge on individual differences and on immunologically important regions of the hantavirus NPs is needed to construct more precise diagnostic tools.
Paper IV: Antibody responses in NE-patients

Based on the results presented in Paper III, we wanted to investigate whether there is a similar individuality to the antibody response in *nephropathia epidemica* patients as we observed in DNA-vaccinated mice. Also, if possible, we wanted to map the cross-reactive epitopes in an effort to resolve the reason for such discrepancies. If these epitopes can be pinpointed, they could have direct diagnostic potential. As earlier hypothesized, the deletion of common epitopes may generate serotype specific antigens, or evenmore, the cross-reactive epitopes might be used as generalized hantavirus antigens. Although such antigens would have limited use in well-equipped medical laboratories, the usefulness when doing epidemiological research or when field-diagnoses are needed is all the more apparent.

Serum samples from 17 NE-patients from the Umeå region were studied. Each sample displayed varying homologous titres, which was quite expected, but it was the comparisons of the individual homologous and heterologous recognition that caught our attention. The recognition of heterologous antigens (SEOV NP and SNV NP) was very varied. Cross-reactions ranged from non-detectable to similar titre levels as towards the homologous protein.

Furthermore, we pursued the epitope-mapping initiated in Paper III using NP deletion mutants and NP alanine-substitution mutants. We discovered that a four amino acid substitution in each of the three NP antigens drastically lowered the antibody recognition of the heterologous antigens. In contrast, the homologous recognition of the substitution mutant
RESULTS AND DISCUSSION

remained equal to the recognition of the corresponding wild type antigen. Therefore, this four aa stretch can be denoted a cross-reactive epitope. We also identified three other epitopes within a 30 aa region of the amino-terminus with different cross-reactive characteristics.

The individualities observed in the heterologous recognition were similar to the discrepancies first observed in DNA-vaccinated mice (III). Although we were able to identify four epitopes with unique cross-reactive characteristics, the findings of these epitopes are not enough to explain the observed differences. The samples from NE-patients were expected to be unique, but the extent of the divergence in cross-reactive recognition was still surprising. The comparative scarceness of shared epitopes between the different hantavirus NPs should not be dismissed, but an additional explanation to the observed differences could be the different HLA haplotypes of the NE-patients. It has previously been demonstrated that the HLA alleles B8 and DRB1*0301 are more frequent in patients with severe nephropathia epidemica (Mäkelä et al., 2002), and the HLA B27 haplotype is associated with a more benign variant of the disease (Mustonen et al., 1998). Similarly, the HLA-B*3501 is connected to more severe HCPS (Kilpatrick et al., 2004). In the light of these findings, the individual differences in epitope recognition should perhaps not be surprising as they suggest a decisive influence of antigen presentation and epitope recognition for the progression of disease. However, in the case of the NE-patients, the different antibody responses could also reflect differences in the infecting PUUV strains. Some studies indicate that such small differences as a single amino acid substitution could affect hantavirus pathogenesis (Ebihara et al., 2000).
RESULTS AND DISCUSSION

Regardless of the reason, these significant differences between individuals should always be considered when evaluating new diagnostic tools and antigens. The idea of constructing peptide antigens to be used to simultaneously diagnose many different hantavirus infections is quite attractive, but the risk of misdiagnosing patients will always be greater when using heterologous antigens. Thus the use of locally derived strains in regional clinics will always be the preferred alternative. However, the methodology based on generalized antigens can be of great use both in epidemiology as well as in POC-tests for facilities and situations where it is not feasible to use locally derived viruses and antigens. In addition, the notion of serotype specific antigens would be particularly useful in areas with several endemic hantavirus species.
4 CONCLUSIONS

This thesis describes the characterization of a local Puumala virus, PUUV Umeå/hu, isolated from a human source. Our findings suggest the importance of using locally derived antigens and sequence information from indigenous viruses in regional clinics. We have also identified regions of the NP as well as pinpointed amino acid residues that are absolutely crucial for NP homotypic interactions, data that we believe will help solve the NP multimerization puzzle. Furthermore, we demonstrate the existence and location of strong amino-terminal B-cell epitopes with distinct cross-reactive characteristics. These epitopes could help distinguish between different hantavirus genera. Apart from increased understanding of the serological relationships of hantaviruses, we believe that these results will be valuable in the development of new serological, genetic, and epidemiological tools.
5 Sammanfattning på svenska

Hantavirus finns över praktiskt taget hela jorden. De orsakar blödarfeber hos människa och varje år läggs uppskattningsvis 150.000 människor in på sjukhus på grund av dessa zoonotiska infektioner. Det finns två typer av sjukdomstillstånd orsakade av hantavirusinfektioner, hemorrhagisk feber med njursyndrom (HFRS) eller hantavirus hjärt/lung-syndrom (HCPS). De två sjukdomarna, som varierar i svårighetsgrad, orsakas av olika typer av hantavirus.

Hantavirus är s.k. "emerging infections", d.v.s. antalet rapporterade fall av hantavirala sjukdomar ökar, nya hantavirus upptäcks och redan kända hantavirus förväntas sprida sig till nya områden. Kunskap om dessa virus och övervakning av dem är därför viktigt ur ett folkhälsoperspektiv.

I Sverige finns ett hantavirus som heter Puumalavirus (PUUV). Det orsakar nephropathia epidemica (NE), sorkfeber, och är en mild variant av blödarfeber. Trots att NE anses vara en mindre allvarlig sjukdom – för att vara en blödarfeber – kan sviterna efter NE bestå i månader, och dödligheten på 0.1-1 % motsvarar några dödsfall per år. Det är olika sorters gnagare som är bärare av dessa hantavirus, och smittan sprids via deras saliv, urin och avföring till oss människor genom inandning av förorenat damm.

I dagsläget är det fortfarande mycket som är okänt vad gäller hantavirus och sjukdomarna de orsakar. Genom att studera dessa virus och förstå hur de t.ex. infekterar, replikerar och orsakar sjukdom kan man bättre hantera de utmaningar som den ökande förekomsten av hantavirus infektioner innebär.


Våra fynd tydliggör komplexiteten hos det immunologiska svaret mot hantavirusinfektioner, och de betonar vikten av att skråddarsy de diagnostiska verktygen samt att utföra klinisk diagnostik på lokala virus. Sammanfattningsvis bidrar våra resultat med värdefull kunskap för utvecklingen av nya serologiska-, genetiska- och epidemiologiska verktyg.
6 ACKNOWLEDGEMENTS

There are so many people I am grateful to, who have helped me with small or large things throughout the years. So, I would like to start by saying a great big THANK YOU to all of you, and especially to the following:

My supervisors: Göran (GB when you’re not around), thank you for unfailing support, fruitful discussions and for handling my stubbornness so well. There would not have been a thesis without you!; and Clas, for all of your help, your cheerfulness and for always making me consider the “doctor”-perspective.

All former members of the FOI virology group: Marlène, Bosse, Lena, Patrik, and Maggan for your expertise, help and support; Anna, for supporting me in all sorts of ways and for being a friend; Therése, for help and great lab-company; Henrik (my badminton-buddy), and Eva, for all the help and for brightening the lab; Anna L (what’s your married name?), for great company on and off work; Anna Ö, for your energy and for making the days at the lab much more fun; Kattis, for… well everything! You’re the best!; Jonas, for all the lab-help, all the fika, and for just being such a great guy, thank you!; Lilla Nina (a.k.a. the handbag-deliverer), for all the help, all the fika-company, and for your great spirit and humor.

Other people I would like to acknowledge are: Per, Göran, and Katrine at the Department of Virology, Umeå University, for their help and support;

“The bacteria” at FOI: Kerstin K, Anna-Lena, Solveig, Laila, and Emelie. Thank you for your expertise and help through the years and just for being there and making the lab such a great place!

Other FOI-folk: Anna M, for always having time to help and for always being such a cheerful pick-me-up; and Marie Lindgren, for always helping out with practical issues.

At the Department of Infectious Diseases, a special thanks to Gunborg who can (and will) fix practically anything.
ACKNOWLEDGEMENTS

Also, I would like to thank supporters and friends outside of work:

Kristin (bruden!), for all the fun, all the parties and your fabulous sense of humor ☺, you’re the greatest! And Linda (vem sover i garderoben?) for being such a good friend and just for being you. Also, thanks to Liza for the TBi-years of studying and partying. Thanks, you guys, for all the partying, cramming, baking (with subsequent fika), “hanging”, and don’t forget the occasional orienteering we’ve shared through the years. You’re the best!

All the members of The Mafia: Helena, Jamilla, Jenny, Lotta, Marie, Sandra, Sofia, and Ullis and naturally all of the kids. Thanks for playdates, “after-works” (even though none of us actually worked ☺), sprit-fester (no way I’m translating that) and theme-parties (I’m not specifying that one either! ☺). You’re great guys!

My family: mamma & pappa, for supporting me through everything; The gingerbread-brothers and their families: Daniel, Ann & my one and only favourite niece Alexandra; Martin & Jenny, thanks for everything. Thanks also to the coolest (and probably most frequently on-line) 84-year old in the world, my grandmother: Mormor du är bäst! Jag hoppas att jag blir som du när jag blir gammal!

My new family: Anders, Christina, Åsa, Tjelvar and little Torunn, for all the help, all the dinners, all the fika, all the baby-sitting, but mostly for welcoming me into the family.

And finally, the light of my life — my boys: Malte and Axel, my pride and joy; and my very own norrlänning Andreas, thanks for understanding me and supporting me always. I love you.

And now, if someone feels forgotten and neglected, please feel free to add your name on the dotted line below ☺:

Thanks a million, ………………., for your fabulous help and support during the course of my Ph.D. You’re the greatest!
7 REFERENCES


REFERENCES

REFERENCES


REFERENCES


REFERENCES


REFERENCES


Hooper, J., Custer, D., Thompson, E. and Schmaljohn, C. (2001a) DNA vaccination with the Hantaan virus M gene protects Hamsters
against three of four HFRS hantaviruses and elicits a high-titer neutralizing antibody response in Rhesus monkeys. J Virol 75(18), 8469-77.


REFERENCES


REFERENCES


64
REFERENCES


nucleocapsid proteins protect mice against challenge in vivo. Viral Immunol 21(1), 49-60.


66
REFERENCES


REFERENCES


68
REFERENCES


REFERENCES


REFERENCES


REFERENCES

recovery from hantavirus cardiopulmonary syndrome. Emerg Infect Dis 10(3), 478-82.