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MOLECULAR GENETICS AND EVOLUTION OF PUUMALA HANTAVIRUS

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ABBREVIATIONS

aa	amino acid(s)
Ab	antibody
Ag	antigen
CR	coding region
cRNA	complementary RNA
DAN	Danish genetic lineage of PUUV
EIA	enzyme immunoassay
FIN	Finnish lineage
Gn, Gc	hantavirus glycoproteins
HFRS	hemorrhagic fever with renal syndrome
HPS	hantavirus pulmonary syndrome
N	nucleocapsid protein
NCR	non-coding region
NE	nephropathia epidemica
nt	nucleotide(s)
NSCA	Northern Scandinavian lineage
ORF	open reading frame
PUUV	Puumala virus
SSCA	Southern Scandinavian lineage
RdRp	RNA-dependent RNA polymerase
RNP	ribonucleoprotein
RT-PCR	reverse transcription – polymerase chain reaction
RUS	Russian lineage
vRNA	viral genomic RNA

Complete list of Puumala virus strains and their abbreviations used in this study are in Appendix I, page 58

SUMMARY

Puumala virus (PUUV) is the causative agent of nephropathia epidemica (NE), a mild form of hemorrhagic fever with renal syndrome. Finland has the highest documented incidence of NE with around 1000 cases diagnosed annually. PUUV is also found in other Scandinavian countries, Central Europe and the European part of Russia. PUUV belongs to the genus *Hantavirus* in the family *Bunyaviridae*.

Hantaviruses are rodent-borne viruses each carried by a specific host that is persistently and asymptomatically infected by the virus. PUUV is carried by the bank voles (*Myodes glareolus*, previously known as *Clethrionomys glareolus*). Hantaviruses have co-evolved with their carrier rodents for millions of years and these host animals are the evolutionary scene of hantaviruses.

In this study, PUUV sequences were recovered from bank voles captured in Denmark and Russian Karelia to study the evolution of PUUV in Fennoscandia. Phylogenetic analysis of these strains showed a geographical clustering of genetic variants following the presumed migration pattern of bank voles during the recolonization of Fennoscandia after the last ice age approximately 10 000 years ago. The currently known PUUV genome sequences were subjected to in-depth phylogenetic analyses and the results showed that genetic drift seems to be the major mechanism of PUUV evolution. In general, PUUV seems to evolve quite slowly following a molecular clock. We also found evidence for recombination in the evolution of some genetic lineages of PUUV. Viral microevolution was studied in controlled virus transmission in colonized bank voles and changes in quasispecies dynamics were recorded as the virus was transmitted from one animal to another. We witnessed PUUV evolution *in vivo*, as one synonymous mutation became repeatedly fixed in the viral genome during the experiment.

The detailed knowledge on the PUUV diversity was used to establish new sensitive and specific detection methods for this virus. Direct viral invasion of the hypophysis was demonstrated for the first time in a lethal case of NE. PUUV detection was done by immunohistochemistry, *in situ* hybridization and RT-nested-PCR of the autopsy tissue samples.

REVIEW OF THE LITERATURE

Discovery of hantaviruses

Hantaviruses are old viruses that have co-evolved with their carrier rodents for millions of years. The human diseases were described already in Chinese writings from the 10th century. The mildest form of hantavirus disease, nephropathia epidemica (NE), was first described in Sweden in 1934 (Myhrman, 1951). An epidemic of NE was reported among Finnish and German troops in Finnish Lapland during the World War II, and in the 1950s "Korean hemorrhagic fever" was described during the Korean War (Earle 1954). Bank voles were suggested as the reservoir of the pathogen by Soviet Union researchers at the end of 1950s. Gradually it became evident that the severe diseases in Far East and in China and the mild diseases in Scandinavia and Eastern Europe were caused by related agents and the term hemorrhagic fever with renal syndrome (HFRS) was introduced (Gajdusek, 1962).

Dr. Ho Wang Lee was the first to identify the causative agent of HFRS. The sera of Korean hemorrhagic fever patients reacted with tissue sections of the striped field mice (*Apodemus agrarius*) (Lee and Lee 1976). Subsequently, the first hantavirus, Hantaan (HTNV), was isolated by passaging it in laboratory-colonized field mice (Lee and Lee 1978). Later this virus was also isolated in cell culture (French *et al.*, 1981). Following that lead, Dr. Markus Brummer-Korvenkontio and co-workers showed in 1980 that lung specimens of the bank vole react with sera from Finnish NE patients (Brummer-Korvenkontio *et al.*, 1980). The first bank vole samples were collected in the vicinity of the village Puumala in Southeast Finland and thus the virus was named Puumala (PUUV). It was first isolated in colonized bank voles (Brummer-Korvenkontio *et al.*, 1980, 1982) and later also in cell culture (Niklasson and LeDuc 1984, Yanagihara *et al.*, 1984, Schmaljohn *et al.*, 1985). In the 1980s two other hantaviruses were isolated: Seoul virus (SEOV) from rats (*Rattus norvegicus* and *Rattus rattus)* (Lee *et al.*, 1982) and Prospect Hill virus (PHV) from meadow voles (*Microtus pennsylvanicus*) (Lee *et al.*, 1982). Yet, the first isolated hantavirus was actually the Thottapalayam virus (TPMV)

carried by shrews (*Suncus murinus*) (Carey *et al.*, 1971), but it was not identified as a hantavirus until the 1990s (Xiao *et al.*, 1994). The next hantaviruses discovered were the Thailand virus (THAIV) (Elwell *et al.*, 1985) and the Dobrava virus (DOBV) that causes severe HFRS in Europe (Avsic-Zupanc *et al.*, 1992, 1995). A turning point in hantavirus research was reached in the 1990s, when the Sin Nombre virus (SNV) causing the disease hantavirus pulmonary syndrome (HPS) was discovered (Nichol *et al.*, 1993, Elliott *et al.*, 1994). Ever since, a number of new hantaviruses have been discovered either through isolation or by sequencing (see Table 1 for summary).

Table 1. List of hantaviruses and their carrier rodents. In bold are the hantavirus species currently recognized by the International Committee on Taxonomy of Viruses.

Virus	Abbr.	Host	Area	Disease (if known)	Reference
Hantaviruses carried by rodents from the family Cricetidae, subfamily Arvicolinae					
Puumala	PUUV	*Myodes glareolus* (bank vole)	Europe	HFRS	Brummer-Korvenkontio *et al.*, 1980
Tula	TULV	*Microtus arvalis* (European common vole)	Europe		Plyusnin *et al.*, 1994
Topografov	TOPV	*Lemmus sibiricus* Lemming	Asia		Plyusnin *et al.*, 1996
Khabarovsk	KHAV	*Microtus fortis* (reed vole)	Asia		Hörling *et al.*, 1996
Prospect Hill	PHV	*Microtus pennsylvanicus* (meadow vole)	North America		Lee *et al.*, 1982, 1985
Isla Vista	ISLAV	*Microtus californicus* (Californian vole)	North America		Song *et al.*, 1995
Bloodland Lake	BLLV	*Microtus ochrogaster* (prairie vole)	North America		Song *et al.*, 1995
Vladivostok	VLAV	*Microtus fortis* (reed vole)	Asia		Kariwa *et al.*, 1999
Muju	MUJV	*Myodes regulus* (royal vole)	Asia		Direct submission to GenBank
Hokkaido	HOKV	*Myodes rufocanus* (red bank vole)	Asia		Kariwa *et al.*, 1995
Fusong		*Myodes rufocanus* (red bank vole)	Asia		Direct submission to GenBank
Hantaviruses carried by rodents from the family Cricetidae, subfamily Neotominae					
Sin Nombre	SNV	*Peromyscus maniculatus* (deer mouse)	North America	HPS	Nichol *et al.*, 1993
New York	NYV	*Peromyscus leucopus* (white-footed mouse)	North America	HPS	Hjelle *et al.*, 1995a, 1995b
El Moro Canyon	ELMCV	*Reithrodontomys megalotis* (western harvest mouse)	North America		Hjelle *et al.*, 1994
Rio Segundo	RIOSV	*Reithrodontomys mexicanus* (Mexican harvest mouse)	South America		Hjelle *et al.*, 1995
Limestone Canyon	LSCV	*Reithrodontomys megalotis* (western harvest mouse)	North America		Sanchez *et al.*, 2001

Hantaviruses carried by rodents from the family Cricetidae, subfamily Sigmodontinae					
Andes	ANDV	*Oligoryzomys longicaudatus* (long-tail pigmy rice rat)	South America	HPS	Lopez *et al.*, 1996; Meissner *et al.*, 2002
Bayou	BAYV	*Oryzomys palustris* (rice rat)	North America	HPS	Morzunov *et al.*, 1995
Black Creek Canal	BCCV	*Sigmodon hispidus* (hispid cotton rat)	North America	HPS	Ravkov *et al.*, 1995
Cano Delgadito	CADV	*Sigmodon alstoni* (cane mouse)	South America		Fulhosrt *et al.*, 1997
Laguna Negra	LANV	*Calomys laucha* (vesper mouse)	South America	HPS	Johnson *et al.*, 1997
Muleshoe	MULV	*Sigmodon hispidus* (hispid cotton rat)	North America		Rawlings *et al.*, 1996
Rio Mamore	RIOMV	*Oligoryzomys microtis* (small-eared pygmy rice rat)	South America		Bharadwaj *et al.*, 1997
Blue River	BRV	*Peromyscus leucopus* (white-footed mouse)	North America		Morzunov *et al.*, 1998
Monongahela	MGLV	*Peromyscus maniculatus* (deer mouse)	North America	HPS	Song *et al.*, 1996
Bermejo	BMJV	*Oligoryzomys chacoensis*	South America		Levis *et al.*, 1997
Lechiguanas	LECV	*Oligoryzomys flavescens* (rice rat)	South America	HPS	Levis *et al.*, 1997
Maciel	MCLV	*Bolomys obscurus* (dark bolo mouse)	South America		Levis *et al.*, 1998; Bohlman *et al.*, 2002
Oran	ORNV	*Oligoryzomys longicaudatus* (long-tail pigmy rice rat)	South America	HPS	Levis *et al.*, 1998; Bohlman *et al.*, 2002
Pergamino	PRGV	*Akadon azarae* (Azara's grass mouse)	South America		Levis *et al.*, 1998; Bohlman *et al.*, 2002
Chochlo		*Oligoryzomys fulvescens* (pygmy rice rat)	N and S America	HPS	Vincent *et al.*, 2000
Calabazo		*Zygodontomys brevicauda* (short-tailed cane mouse)	South America	HPS	Vincent *et al.*, 2000
Araraquara		*Bolomys lasiurus* (hairy-tailed bolo mouse)	South America	HPS	Johnson *et al.*, 1999
Hantaviruses carried by rodents from the family Muridae, subfamily Murinae					
Hantaan	HTNV	*Apodemus agrarius* (striped field mouse)	Asia	HFRS	Lee *et al.*, 1978
Seoul	SEOV	*Rattus norvegicus* (Norway rat)	Global	HFRS	Lee *et al.*, 1982
Dobrava	DOBV	*Apodemus flavicollis* (yellow-necked mouse)	Asia	HFRS	Avsic-Zupanc *et al.*, 1992, 1995
Thailand	THAI	*Bandicota indica* (bandicoot rat)	Asia		Elwell *et al.*, 1985
Amur	AMRV	*Apodemus peninsulae* (Korean field mouse)	Asia	HFRS	Yashina *et al.*, 2001, Lokugamage *et al.*, 2002
Soochong	SOOV	*Apodemus peninsulae* (Korean field mouse)	Asia		Baek *et al.*, 2006
Da Bie Shan	DBSV	*Niviventer confucianus* (Chinese white-bellied rat)	Asia		Wang *et al.*, 2000
Saaremaa	SAAV	*Apodemus agrarius* (striped field mouse)	Asia	HFRS	Plyusnin *et al.*, 1997; Nemirov *et al.*, 1999
Hantavirus carried by an insectivore (non-rodent)					
Thottapalayam	TPMV	*Suncus murinus* (shrew)	Asia		Carey *et al.*, 1971; Xiao *et al.*, 1994

Taxonomy of hantaviruses

PUUV belongs to the genus *Hantavirus*, family *Bunyaviridae* that is comprised of five genera: *Orthobunyavirus, Hantavirus, Nairovirus, Phlebovirus* and *Tospovirus* (Nichol *et al.*, 2005). All viruses in these genera have a similar virion structure, genome

organization and replication strategy. They have 3 molecules of negative or ambisense ssRNA as their genome. These segments are designated L (large), M (medium) and S (small) and their total length is 11-19 kb. The L, M and S segments encode, respectively, the viral RNA-dependent RNA polymerase (L protein), two envelope glycoproteins (Gn and Gc) and the nucleocapsid protein (N). Each RNA segment is non-covalently circular and the terminal nucleotides are complementary and form a base-paired panhandle-like structure. The virions are spherical and they are comprised of enveloped ribonucleocapsids (Figure 1). Some viruses in this family also encode additional nonstructural proteins in the M (NSm) or S segment (NSs). Viral replication occurs in the cytoplasm, and the virions bud into the Golgi cisternae from where they are transported to the plasma membrane and released (Nichol *et al.*, 2005).

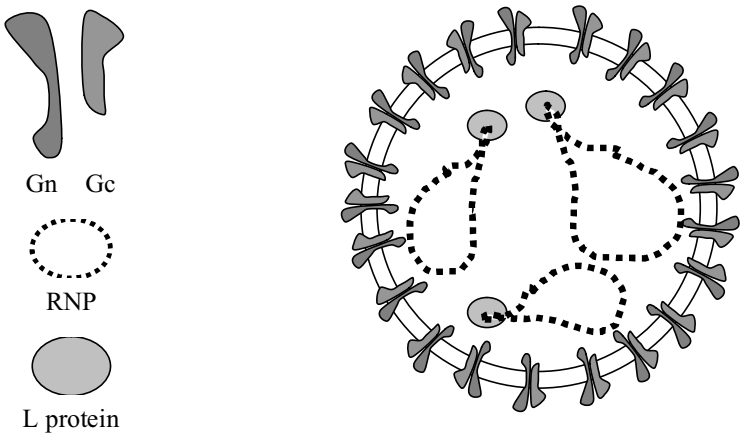

Figure 1. Schematic structure of a hantavirus.

The genus *Hantavirus* includes 22 officially recognized virus species, and in addition to these, at least 19 tentative hantavirus species (see Table 1 for a summary). These viruses are a prime example of emerging pathogens that have attracted more and more attention in the last decades. Unlike all other viruses in the family *Bunyaviridae*, hantaviruses lack an arthropod vector. Instead, they are transmitted from rodent to rodent (and occasionally

also from rodent to human) via indirect contacts. Each hantavirus is carried by a specific host and currently there are 50 different rodent species that are reported to be infected by hantaviruses (Zeier *et al.*, 2005). Human infections are incidental and human-to-human transmission has only been reported for the Andes virus (Martinez *et al.*, 2005). Hantaviruses cause two types of human diseases, HFRS and HPS, and some of the viruses are apparently non-pathogenic to humans. In the rodent hosts, the viruses cause a life-long persistent and asymptomatic infection (Plyusnin *et al.*, 1996, Nemirov *et al.*, 2004a). Hantaviruses are thought to co-evolve with their rodent hosts and this is reflected in their phylogeny (Figure 2).

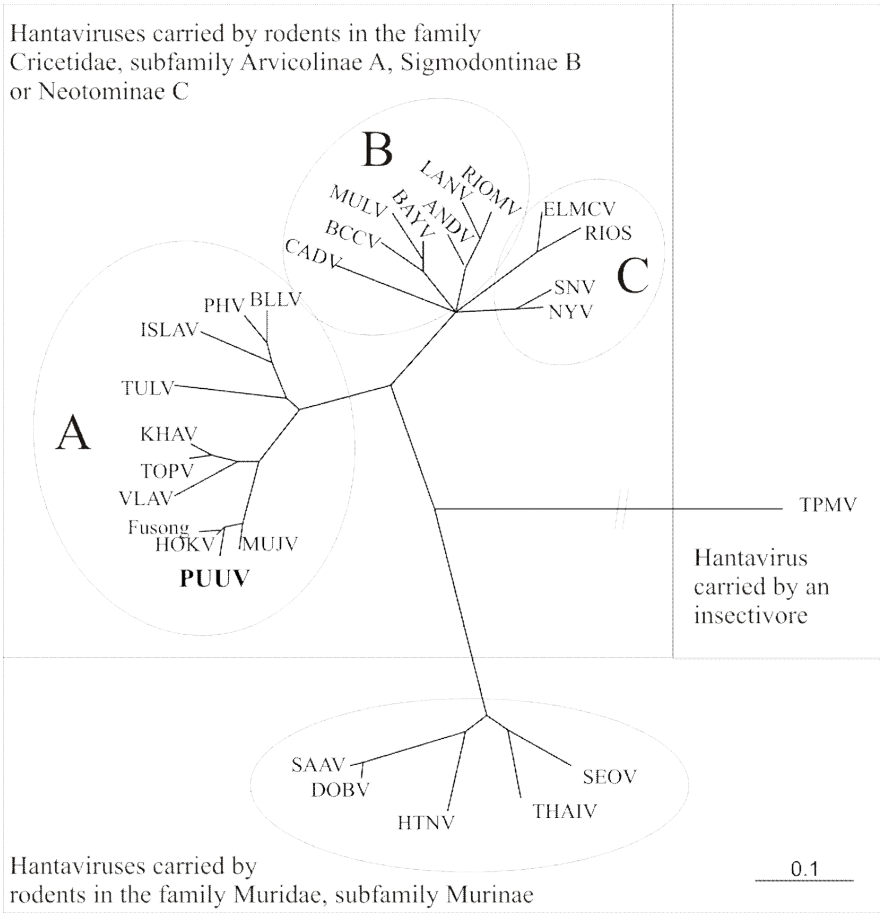

Figure 2. Phylogenetic tree of hantavirus N protein sequences (calculated using TreePuzzle) showing their grouping following the carrier rodents. The abbreviations are described in table 1.

The International Committee on Taxonomy of Viruses has formulated four criteria to define hantavirus species. First, the different species should have a different primary reservoir, i.e. they are found in a unique ecological niche. Second, the aa difference of the complete glycoprotein precursor protein and the nucleocapsid protein sequences is at least 7%. Third, the difference in two-way cross-neutralization test is at least four-fold. Fourth, different hantavirus species do not form reassortants naturally with other species (Nichol *et al.*, 2005).

Distribution of Puumala virus

PUUV has been isolated and sequences have been recorded from several European countries (Figure 3): Austria (Aberle *et al.*, 1999, Plyusnina *et al.*, 2006), Belgium (Escutenaire *et al.*, 2001), Croatia (Cvetko *et al.*, 2005), Denmark (**I, IV**), Estonia (Golovljova *et al.*, 2002), Finland (Vapalahti *et al.*, 1992, Plyusnin *et al.*, 1995, 1997, 1999), France (Plyusnina *et al.*, 2007), Germany (Pilaski *et al.*, 1994, Heiske *et al.*, 1999, Bahr *et al.*, 2006, Essbauer *et al.*, 2006), Norway (Lundkvist *et al.*, 1998), Russia (Stohwasser *et al.*, 1990, Plyusnin *et al.*, 1994, 1995, **I**, Dekonenko *et al.*, 2003), Slovenia (Avsic-Zupanc *et al.*, 2007), and Sweden (Hörling *et al.*, 1996, 1995, Johansson *et al.*, 2004). The largest collection of hantavirus sequences is available for PUUV. Currently there are 45 complete S segment sequences and 125 partial S segment sequences deposited in the GenBank. Furthermore, 9 complete M segment sequences, and 5 complete L segment sequences are available. In addition to *bona fide* PUUV sequences, there are two complete S segment sequences from the closely related Hokkaido virus (HOKV) that is carried by *Myodes rufocanus,* the red bank vole. Recently, 4 complete S segment sequences of another related hantavirus, Muju (MUJV) were also deposited in the GenBank. This virus is carried by *Myodes regulus*, the royal vole.

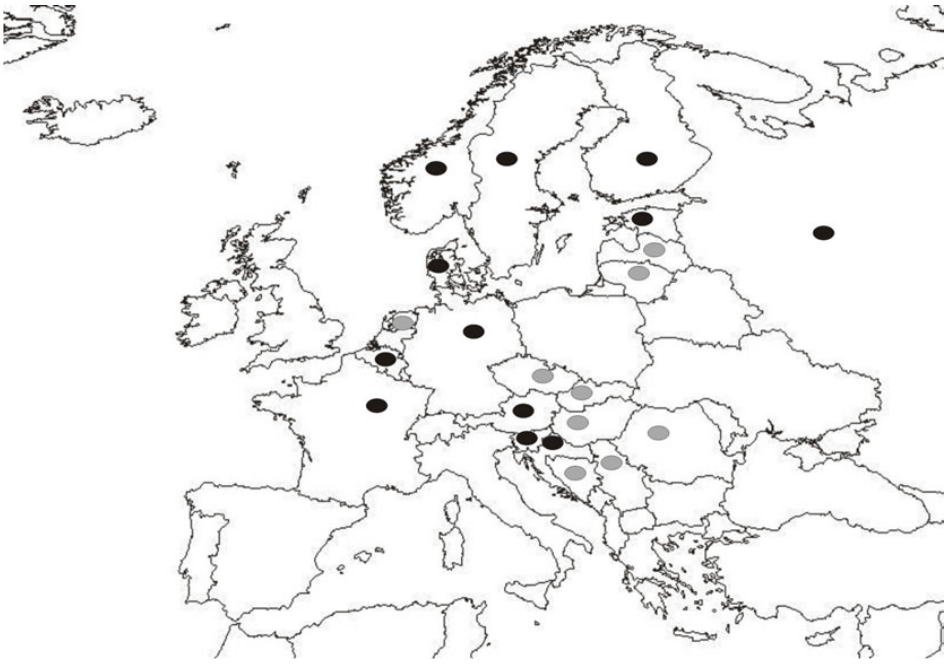

Figure 3. European countries where PUUV is found. Black dots mark countries from where PUUV genome sequences are available. Grey dots show countries where human PUUV infections have been found.

Mechanisms of hantavirus evolution

The persistently infected rodents are the evolutionary scene for hantaviruses (Plyusnin *et al.*, 1996). Genetic analysis has suggested that the main mechanism leading to hantavirus diversification is the genetic drift, i.e. the gradual accumulation of genomic changes such as nucleotide substitutions, deletions and insertions (Plyusnin *et al.*, 1995, Rowe *et al.*, 1995, Lundkvist *et al.*, 1998). Similarly to many other RNA viruses, hantaviruses appear as populations of closely related genetic variants, i.e. quasispecies (Plyusnin *et al.*, 1995, 1996, Lundkvist *et al.*, 1997, Feuer *et al.*, 1999). Although this would provide the possibility for rapid evolution, hantaviruses seem to evolve slowly. They have adapted to their rodent hosts a long time ago and there is little selection pressure for change in these animals (Lundkvist *et al.*, 1992). Strong purifying selection is apparent as high level of strain variation at the nt level is translated only to a low variation at the aa level (Hjelle *et*

al., 1995). Hantavirus evolution may also be driven by positive selection in situations requiring fast adaptation. This has been shown in the adaptation of PUUV to cell culture, which was accomplished through an expanded quasispecies diversity and fixation of nt substitutions in the non-coding (NCR) regions of the S segment (Lundkvist *et al.*, 1997). Two point mutations (one silent and one leading to aa substitution) in the coding region of the L segment were also found (Nemirov *et al.*, 2003). These minimal changes in the genotype were translated to clearly distinct phenotypes. The cell culture –adapted PUUV variant was neither infectious to bank voles (Lundkvist *et al.*, 1997) nor the Cynomolgus macaques used as a non-human primate model of NE (Groen *et al.*, 1995). Another situation accompanied by fast adaptation is host switching. In general, hantaviruses are firmly associated with their rodent hosts. Yet sometimes they have apparently spread and colonized new hosts as suggested for MGLV/NYV (Morzunov *et al.*, 1998), TOPV/KHAV (Vapalahti *et al.*, 1999) and DOBV/SAAV (Nemirov *et al.*, 2002)

The life-long hantavirus infection of the carrier rodents provides the possibility of co-infections and thus recombination or reassortment between different viral strains. These mechanisms are called the genetic shift. Hantavirus reassortment was first demonstrated for Sin Nombre virus (Li *et al.*, 1995). The data showed that especially the reassortment of the M segment may occur. Reassortment of hantaviruses of the same genotype has also been demonstrated *in vitro* fairly frequently (Rodriquez *et al.*, 1998). In contrast, reassortment between two hantavirus genotypes was infrequent, and importantly, these reassortants were unstable and deleterious. Genetic shift in hantaviruses may also happen through recombination. This was first demonstrated for Tula virus (Sibold *et al.*, 1999, Plyusnin *et al.*, 2002) and later also for Puumala virus (**II**) and Hantaan virus (Chare *et al.*, 2003). Recombination is an important feature in the evolution of many RNA viruses (Worobey *et al.*, 1999). Furthermore, it is known that RNA viruses vary greatly in their ability to undergo recombination (Posada *et al.*, 2002). The highest rates of recombination are those of retroviruses such as HIV (Malim *et al.*, 2001), whereas the lowest rates are observed for the negative-stranded RNA viruses (Chare *et al.*, 2003). They have their genomes packaged into RNP structures, which may limit their ability to

recombine. Most recombinants are also likely to be deleterious and thus they will be removed by purifying selection as seen, e.g. in coronaviruses (Banner and Lai, 1991).

Hantaviral genes and proteins

Similarly to other hantaviruses, PUUV has a tripartite genome of negative-stranded RNA. The S segment encodes the nucleocapsid protein (N) (Schmaljohn *et al.*, 1986). The hantaviral nucleocapsid protein has many activities in the viral RNA replication, encapsidation and virus assembly (Kaukinen *et al.*, 2005). It has a major role in the protection of the RNA genome through formation of the ribonucleoprotein complexes (RNP) (Flint *et al.*, 2000). The N protein has to interact with both the viral RNA and other N protein molecules to form these RNP structures. The terminal non-coding regions of the hantaviral vRNA molecules have been shown to contain a unique binding site for the N protein (Severson *et al.*, 1999, Mir and Panganiban, 2004). The RNA-binding domain of the HTNV N protein has been mapped to the middle part of the protein (aa 175 to 217) and this region is highly conserved among hantaviruses (Xu *et al.*, 2002). The homotypic interactions of hantavirus N proteins have been mapped to the amino-terminal and carboxy-terminal regions (Alfadhli *et al.*, 2001, Kaukinen *et al.*, 2003, Yoshimatsu *et al.*, 2003), and this interaction has been further characterized using *in silico* modeling and point mutagenesis (Alfadhli *et al.*, 2002, Kaukinen *et al.*, 2004, Alminaite *et al.*, 2006). The crystal structure of a partial SNV N protein was solved very recently showing a coiled-coil structure as predicted earlier (Boudko *et al.*, 2007).

The hantavirus N protein has been shown to interact with cellular proteins such as actin (Ravkov *et al.*, 1998) and the apoptosis regulator Daxx (Li *et al.*, 2002). Furthermore, yeast two-hybrid assays have suggested that it can also interact with Ubc-9 and SUMO-1 that are involved in the sumoylation reactions in the host cell (Kaukinen *et al.*, 2003, Maeda *et al.*, 2003). It has been speculated that the N protein–SUMO-1 interaction would retain the N protein in the cytoplasm, but further studies are needed to confirm this. The N protein also interacts with the other viral proteins, the RNA-dependent RNA polymerase (Kukkonen *et al.*, 2004a, 2004b) and the envelope glycoproteins Gn and Gc.

The hantaviral L protein is presumably associated with the RNPs and thus it can initiate viral RNA synthesis immediately after the virus entry into a cell (Schmaljohn and Dalrymple, 1983). Studies using minigenome systems have shown that only functional hantaviral L and N proteins are needed for RNA replication and transcription (Flick *et al.*, 2003). Furthermore, the N protein is highly immunogenic and B-cell epitopes have been mapped in its N-terminal third (Lundkvist *et al.*, 1995, Vapalahti et al, 1995, Yoshimatsu *et al.*, 1996) while T-cell epitopes are in the central region of the protein (Ennis *et al.*, 1997, van Epps *et al.*, 1999, 2002, Park *et al.*, 2000).

The hantavirus M segment encodes the two envelope glycoproteins Gn and Gc that are synthesized first as a precursor polypeptide, and later cotranslationally cleaved to yield the two mature proteins (Löber *et al.*, 2001). They are type I integral membrane proteins with their C-terminal domains in the cytoplasm (Spiropoulou, 2001). The glycoproteins Gn and Gc are targeted to the endoplasmic reticulum where they form a heterodimer (Ruusala *et al.*, 1992). They are further transported to the Golgi compartment, where they are glycosylated (Schmaljohn *et al.,* 1986, Shi and Elliott, 2002). The signal sequence targeting these proteins to the Golgi complex is most probably located in the cytoplasmic tail of the Gn protein, similarly to Uukuniemi virus (UUKV) (Andersson *et al.*, 1997). In the virion, they are embedded in the envelope and exposed on the viral surface. Presumably they are responsible for binding of the virus to the target cells although this has not been shown directly yet. The glycoproteins are highly variable and they are the target for neutralizing antibodies (Dantas *et al.*, 1986, Ruo *et al.*, 1991, Lundkvist *et al.*, 1993a, 1993b, 1995).

The L segment encodes the putative viral RNA-dependent RNA polymerase (RdRp) (Stohwasser *et al.*, 1991). It should contain several enzymatic activities such as endonuclease, transcriptase, replicase and RNA helicase activity (Johnsson and Schmaljohn, 2001). Conserved motifs common to other RdRps of negative-stranded RNA viruses have been recognized (Kukkonen *et al.*, 1998, Nemirov *et al.*, 2003). The L protein is located in the perinuclear region of the cell and it is associated with cellular membranes (Kukkonen *et al.*, 2004). Indeed, it is known that the RNA synthesis of

positive-strand RNA viruses is associated with cellular membranes (Salonen *et al.*, 2005), and this has been suggested for negative-strand RNA viruses as well.

Hantaviral replication cycle

The replication cycle of hantaviruses is comprised of virus attachment, entry, transcription, translation, RNA replication, virion assembly and release of progeny viruses. Similarly to many other viruses (Schneider-Schaulies, 2000), hantaviruses use integrins as their receptors for attachment to the host cell surface (Gavrilovskaya *et al.*, 1998, 1999, 2002, Mackow *et al.*, 2002). More recently, additional receptors have been recognized, but their role is yet to be defined (Kim *et al.*, 2002, Mou *et al.*, 2006). Integrins are essential cell adhesion molecules mediating cell-cell and cell-extracellular matrix interactions (Hynes, 2002). Interestingly, the pathogenic hantaviruses use β3-integrins as their receptors while the non-pathogenic hantaviruses seem to use β1-integrins (Gavrilovskaya *et al.*, 2002). There are two types of β3-integrins: αIIbβ3 and αVβ3. αIIbβ3 integrin is mainly expressed on the surface of platelets, and αVβ3 on the surface of endothelial cells, smooth muscle cells, monocytes, and platelets. This distribution is in agreement with the studies showing that pathogenic hantaviruses can infect endothelial cells and monocyte/macrophages in culture (Pensiero *et al.*, 1992, Temonen *et al.*, 1993). Following attachment, hantaviruses enter the cells via clathrin-dependent receptor-mediated endocytosis (Jin *et al.*, 2002). The site and mechanism of uncoating are unknown, but this step seems to be low-pH-dependent (Jin *et al.*, 2002).

Primary transcription of viral mRNAs is initiated shortly after the release of viral RNP into the cytoplasm and it is started by the viral RdRp in the virion. The viral polymerase generates primers for viral mRNA synthesis from cellular mRNAs by the “cap snatching” mechanism. “Prime-and-realign” transcription ensures that copies of the viral RNA have exact 5’-end sequences (Jonsson and Schmaljohn, 2001). As soon as the viral mRNAs are synthesized, the translation of viral proteins starts. The S and L segment mRNAs are translated on free cytoplasmic ribosomes and the M segment mRNA on membrane-bound ribosomes. The template for viral replication is an exact complementary copy of vRNA,

i.e. antigenomic RNA (cRNA). The newly made vRNA copies increase the mRNA production and synthesis of viral proteins (Jonsson and Schmaljohn, 2001). The hantaviral glycoproteins presumably determine the site of virus assembly and maturation, although the mechanism is currently unknown. Hantavirus particles mature by budding into vesicles within the Golgi apparatus (Petterson *et al.*, 1991). These vesicles are transported to the plasma membrane and released. The first released hantavirus particles appear 24 hours post-infection in cell culture (Kariwa *et al.*, 2003). It has also been suggested that certain Sigmodontinae/Neotominae-carried hantaviruses actually mature at the plasma membrane instead of the Golgi apparatus (Ravkov *et al.*, 1997, Spiropoulou *et al.*, 2003, Shi and Elliott, 2004). The mature hantavirus particles are spherical and they have an average diameter of 120 nm (range 78-210 nm).

PUUV life cycle in nature

PUUV infects the bank voles persistently. The virus can be detected in different organs of the animals, and the highest viral loads are found in the lungs (Yanagihara *et al.*, 1985, Netski *et al.*, 1999). The bank voles shed the virus in the urine, feces and saliva for months (Gavrilovskaya *et al.*, 1990), and PUUV can survive outside the host in rodent excreta at least 2 weeks at room temperature (Kallio *et al.*, 2006). Virus transmission from one animal to another occurs horizontally (Meyer and Schmaljohn, 2000, Kallio *et al.*, 2006). Maternal antibodies protect the newborn animals for up to 80 days (Kallio *et al.*, 2006b), and the maturation as well as the breeding success of these animals are enhanced. In Finland, the density of the rodents varies cyclically with a peak occurring every 3-4 years. The number of clinical NE cases follows these cycles (Vapalahti *et al.,* 2003).

Clinical features of NE

Hantaviruses cause two types of human disease, HFRS and HPS. PUUV causes a mild form of HFRS named NE. Approximately 150 000 HFRS cases occur worldwide annually while around 1000 HPS cases have been reported so far. PUUV is the most

common cause of HFRS found all over Europe. PUUV and NE are highly endemic in the Nordic countries, and in Finland an average of 1000 new cases is diagnosed every year (Brummer-Korvenkontio *et al.*, 1999). Most PUUV infections remain undiagnosed, and the clinical cases vary from mild to lethal. This disease has an acute onset of fever and headache followed by gastrointestinal symptoms, impaired renal function, and blurred vision. The severe cases are characterized by renal failure, circulatory shock and hemorrhage (Settergren 2000). Recovery is usually spontaneous, and mortality is less than 0.1% (Brummer-Korvenkontio *et al.*, 1999). Long-term sequelae are rare, but hypertension and hypertensive renal disease have been reported (Mäkelä *et al.*, 2000, Miettinen *et al.*, 2006). In addition, PUUV may infect the pituitary gland leading to hypophyseal insufficiency that needs to be treated with hormone-replacement therapy. A lifelong immunity remains after the infection and hantavirus antibodies can be detected decades after the infection (Settergren *et al.*, 1991). There is a genetic susceptibility for severe courses of NE associated with human leukocyte antigen B8 haplotype (Mustonen *et al.*, 1996), whereas HLA B27 correlates with the mild course of the disease (Mustonen *et al.*, 1998).

Pathogenesis of PUUV infection

Despite many years of hantavirus research, the pathogenesis of these viruses is still poorly understood. A key feature seems to be increased capillary permeability. Endothelial cells and monocytes are thought to be the main target cells for hantaviruses, but the infection alone has no cytopathic effect on these cells (Temonen *et al.*, 1993). In histological studies, the viral antigens are primarily found within capillary endothelium throughout various tissues. PUUV antigen has been detected in tissues only rarely in kidney biopsies in tubular epithelial cells with focal distribution (Groen *et al.*, 1996), or in autopsy tissue samples (Hautala *et al.*, 2002). Typical histological findings in the kidney biopsies of NE patients are acute tubulointerstitial nephritis, interstitial edema, inflammatory cell infiltrations, tubular epithelial and luminal alterations and slight glomerular changes (Mustonen *et al.*, 1994, Temonen *et al.*, 1996). The pathological findings in lethal cases of NE include hemorrhages in kidneys, endomyocardium and

pituitary gland (Linderholm *et al.*, 1991, Valtonen *et al.*, 1995). Pulmonary edema and venous congestion in other organs including liver, gastrointestinal tract and brain have also been detected in these studies.

The infiltrating cells found in PUUV-infected tissues are plasma cells, monocytes/macrophages, polymorphonuclear cells and lymphocytes, and the predominant ones are the CD8+ T-cells. Increased expression of cytokines, especially tumor necrosis factor (TNF)-α, has been detected at sites of injury (Temonen *et al.*, 1996). Furthermore, increased plasma levels of TNF-α, interleukin(IL)-6 and IL-10 are reported in NE patients (Linderholm *et al.*, 1996) as well as urinary excretion of IL-6 (Mäkelä *et al.*, 2004). Similarly, Cynomolgus macaques infected with wild-type PUUV also demonstrated IL-6, IL-10 and TNF-α responses (Klingström *et al.*, 2002). These data suggest that the immune response plays a critical role in the pathogenesis of PUUV infection (Terajima *et al.*, 2004). The hypothesis is that hyperactivated PUUV-specific T-cells attack endothelial cells presenting PUUV epitopes thus causing damage to the capillary endothelium. The molecular mechanisms of this damage remain unknown. Recently, the evidence for pro-apoptotic properties of hantaviruses has accumulated both in cell culture experiments (Markotic *et al.*, 2003, Li *et al.*, 2004, 2005) and in HFRS patients (Klingström *et al.*, 2006, Liu *et al.*, 2006).

Diagnosis and treatment

Diagnosis of PUUV infections is based on serology, on the detection of serum IgM antibodies or low-avidity IgG antibodies to PUUV (Vapalahti *et al.*, 1996), and these are present already during the first days of the disease. A widely used method is an immunofluorescence assay based on viral antigen grown in cell culture, and enzyme immunoassays based on recombinant nucleocapsid proteins are also used. Alternatively, a rapid point-of-care immunochromatographic IgM test may be applied (Hujakka *et al.*, 2001). Typical laboratory findings include proteinuria, an elevated serum creatinine concentration and thrombocytopenia (Settergren 2000). No specific treatment is currently available, and maintaining fluid balance is critical in the supportive treatment. Severe

cases of NE may require dialysis. Ribavirin has been shown to have anti-hantaviral effects and it may decrease mortality and severity of HFRS symptoms (Huggins *et al.*, 1991).

AIMS OF THE STUDY

- To analyze the genetic diversity and geographic distribution of PUUV
- To elucidate the mechanisms of PUUV evolution
- To develop new detection methods of PUUV in clinical samples and use these in studying pathogenesis of NE in patients

MATERIALS AND METHODS

The methods are described in detail in the articles referred to by their roman numerals.

Method	**Described in**
Animals	V
Antibodies	III
Cloning and sequencing	I-V
Enzyme immunoassay	IV
Immunoblotting	I, IV
Immunohistochemistry	III
Phylogenetic analysis	
PHYLIP	I, II, IV, V
SimPlot	I, II
SplitsTree	II
TreePuzzle	II
Wisconsin package (GCG)	I, II , IV
RNA extraction	
From bank vole tissues	I, II, IV, V
From paraffin-embedded tissues	III
RNA secondary structure prediction	V
Rodent trapping	I, IV
RT-PCR	I-V
Sequence analysis	
Multiple sequence alignment	I, II, IV, V
Sequence handling with Bioedit	IV, V
Sequence handling with SeqApp	I, II
Tissue preparations	III
Virus infection	V

RESULTS AND DISCUSSION

Evolutionary history of PUUV in Northern Europe

After the last ice-age, Fennoscandia was deglaciated appr. 8000-13000 years ago. During the deglaciation, the area was recolonized by a plethora of plant and animal species (Jaarola *et al.*, 1999). The animals, including the bank voles, had survived the ice-age in glacial refugia outside the ice-sheet. One refugium was established south of the ice-sheet in continental Europe, a second refugium was in the east of the current territory of European Russia, and possibly a third refugium was located along the sea coast in Northern Scandinavia (Hewitt 1999, Jaarola *et al.*, 1999, Fedorov and Stenseth, 2001). While retreating from Fennoscandia, the Late Weichselian continental glacier left two potential immigration routes for flora and fauna to recolonize the uncovered land. There was a southern route via present Denmark and Southern Sweden when there still was a land bridge connecting them, and an eastern route via present Russia and Finland. Bank vole populations following these two routes met in central Sweden, forming a contact zone (Limes Norrlandicus), which is still only about 50 km wide. Two types of bank voles (northern and southern) are found in Fennoscandia on different sides of the contact zone. The northern type is carrying mtDNA that originates from a different species, the red vole (*Myodes rutilus*) (Hörling *et al.*, 1996). Studies have shown that also the PUUV strains on different sides of this zone form two distinct phylogenetic lineages (Hörling *et al.*, 1996; Lundkvist *et al.*, 1998), thus supporting the hypothesis of hantavirus-host co-evolution (Plyusnin and Morzunov, 2001, Nemirov *et al.*, 2004). This study was started by collecting PUUV strains from two sites along the postulated immigration routes: Denmark in the southern route and Russian Karelia in the eastern route.

Ninety bank vole tissue samples were collected at three different locations in Russian Karelia. Twelve samples (13%) were determined positive for PUUV N antigen. One positive sample from each of the three locations was selected for amplification of full-length S segment and partial M segment sequences. Five percent (8/152) of the samples collected in Denmark back in 1990 were Ab-positive, but we were able to recover

sequences from only one sample. This was most probably because of suboptimal storage. Thus, two additional Ab-positive bank voles originating from another collection trip in 2001 (**IV**) were added to the analyses, and the S and M segment sequences were recovered from these two. Altogether six new PUUV strains were recovered and designated as Puumala/Karhumäki/Cg117/95, Puumala/Gomselga/Cg4/95, Puumala/Kolodozero/Cg53/95, Puumala/Fyn/Cg19/90, Puumala/Fyn/Cg47/00 and Puumala/Fyn/Cg131/00. GenBank accession numbers of all the sequences used in this study are given in Appendix I.

The S segment sequences of the Russian Karelian strains were 1828-1832 nucleotides (nt) in length, and they all carried a single open reading frame (ORF) of 1302 nt encoding the 433 amino acid (aa) N protein. The Russian Karelian strains showed highest similarity to Finnish strains. The values were 91-93% on the nt level and 96-99% on the aa level. Other PUUV strains were 75-84% identical on nt level and 92-97% identical on aa level. The PUUV strains from the parts of European Russia were the closest to the Finnish and Russian Karelian strains. These results were confirmed by analyses of the M segment nt sequences and deduced Gc aa sequences, and similarities to the Finnish strains were 89-94% and 97-100%. Furthermore, the Russian Karelian strains shared aa fingerprints typical of Finnish strains both in the deduced aa sequence of the N protein (Met_{262}, Asp_{309} and Phe_{388}) and of the Gc protein (Val_{603}) (**I**: Fig. 2).

The Danish PUUV S segment sequences were 1832-1858 in length carrying the ORF for the 433 aa N protein. These strains did not display a particularly close relatedness to any other PUUV strain. The identity of the S segment sequence to all other PUUV strains on the nt level was 74-78% and on the aa level 92-97%, and for the M segment nt and Gc aa sequences 80-84% and 91-97%. No aa signatures were shared with other PUUV strains, instead the Danish PUUV strains had their own specific markers in the N protein (Asp_{272} and Ala_{305}) and in the Gc protein (Leu_{557}) (**I**: Fig. 2). Interestingly, in the Gn sequence, we found one marker shared by the S-SCA (Southern Scandinavian) and Danish strains (Asn_{667}).

Phylogenetic trees were calculated for the complete coding region of the S segment (**I**: Fig. 3A) and partial M segment sequences (**I**: Fig. 3B). PUUV strains formed seven lineages showing typical geographical clustering. The Russian Karelian strains were placed within the Finnish (FIN) lineage with high bootstrap support, suggesting that they share a common recent ancestor. Thus the area that includes southern and central Finland and Russian Karelia was most probably recolonized by the same stream of post-glacial bank vole immigrants.

The Danish PUUV strains formed a distinct genetic lineage on the phylogenetic tree (**I**: Figs. 3A and 3B). A detailed analysis of the Danish sequences was needed to reveal possible evolutionary connections to other PUUV strains. In a similarity plot (**I**: Fig. 4), a higher similarity to the NSCA (Northern Scandinavian) lineage was seen at nt 650-850, and at nt 1050-1250 a higher similarity to the SSCA lineage was detected. These two connections were also supported in phylogenetic trees calculated for these regions of the S segment (**I**: Figs. 4B and 4D). Especially the Danish strain Fyn19 appeared particularly close to the Norwegian strain Eidsvoll. Thus it seems that Danish PUUV strains have something in common with both NSCA and SSCA lineages. These two lineages are associated with two distinct bank vole populations, and this has been considered to support the bi-directional spreading of PUUV into Fennoscandia after the last ice age (Hörling *et al.,*1996). However, the phylogeographical pattern of bank voles is perhaps not that simple, since the Danish population might actually include bank voles of the north-eastern lineage, in addition to the southern lineage of bank voles (Jaarola *et al.,*1999). One possibility is that the northern type of bank voles and virus may have been imported from Northern Scandinavia along with shipped timber, which has been a common practice for hundreds of years. Furthermore, the origin of the NSCA PUUV lineage remains unknown, since no relatives have been found either on the eastern route (Russian Karelian or Finnish strains) or on the southern route (Danish or SSCA strains). It has been speculated, that there might have been local refugia in Northern Scandinavia (Fedorov and Stenseth, 2001) in an area that remained ice-free during the last ice-age (Ignatius *et al.,* 1981) and this could be the original site of NSCA PUUV lineage.

Characterization of PUUV in Denmark

The mosaic-like structure of the S segment of the Danish lineage might indicate recombination event(s) of the precursors of the ancestral lineages. This structure could also be explained by selective preservation of different regions of the genome. In order to study the Danish PUUV strains in more detail, a second trapping expedition was organized. Small mammals were trapped in three locations on the island of Fyn and also in three locations on Jutland, south of Aarhus (**IV**: Fig. 1). Altogether 310 small mammals were trapped including 188 bank voles, and lung samples were available from 159 voles. 305 rodent heart-extract samples were first screened for antibodies (Ab) by EIA using both PUUV- and DOBV-N antigens (Ag). PUUV Ab–positive (OD > 0.150) or borderline reactive (OD 0.050-0.150) samples were then analyzed with immunoblotting, which confirmed 11 samples positive for PUUV-reactive IgG (**IV**: Table 2). All bank vole lung tissue samples were also screened for the viral N-Ag by immunoblotting but only five positive samples were found. The 11 Ab-positive samples were all screened for the viral genome by RT-PCR, and seven positive were found (**IV**: Table 2). The Ab levels in bank voles correlated with the amount of viral RNA and N-Ag detected in lung tissue. At low Ab levels both viral RNA and N-Ag were undetectable. With rising Ab levels, first the viral RNA test and then the N-Ag WB-test became positive. This result is different from a study on Black Creek Canal virus (BCCV) in *Sigmodon hispidus* (cotton rat), in which it was shown that in the acute phase low Ab titers in blood are accompanied by clearly detectable viral RNA in tissues (Hutchinson *et al.,*1998). Later, in the chronic phase, high Ab titers and mostly undetectable viral RNA were seen. In general, hantaviruses are thought to infect their rodent hosts persistently, but with variable kinetics and dynamics (Meyer and Schmaljohn, 2000). Conclusions based on our study are limited by the low number of positive samples, and thus further studies are needed to understand PUUV dynamics in rodents.

Hantaviruses typically have a higher seroprevalence in the older animals than the young ones (Bernshtein *et al.,* 1999). This was also true in our study although the number of PUUV- positive bank voles was small (**IV**: Table 3). Interestingly, PUUV was only found

on the island of Fyn, while rodents trapped in the Jutland mainland were all negative for PUUV. Still, bank voles can be found all over Denmark at appropriate landscapes, and in fact, the Danish islands (Fyn, Zealand and Lolland) as well as the peninsula of Jutland, all share a similar landscape. The incidence of NE is low in Denmark, and most of the patients caught their infection on the island of Fyn (H.K. Andersen, unpublished data/ **IV**). On this island, there are at least two clusters of cases at the southeastern and western parts of Fyn (**IV**: Fig. 1). In these locations, PUUV prevalence in the bank voles was determined to be 22% and 7%, respectively. The overall seroprevalence of 14% is rather low compared to situation in northern locations (Brummer-Korvenkontio *et al.,* 1982). Some human cases of NE are also found on the mainland and other parts of Denmark, and thus PUUV should also circulate there, although we did not find such samples in this study. However, other pathogenic hantaviruses may also cause human infections in Denmark, and at least SAAV has been found in Denmark on the island of Lolland (Nemirov *et al.,* 2004).

Partial M segment sequences (nucleotides 2168-2659) were recovered from the seven RT-PCR -positive rodents captured on Fyn. Two PUUV strains from Denmark, Fyn/19Cg/90 (Fyn19 for short) and Fyn/47Cg/00 (Fyn47), were described earlier (**I**). Sequences recovered from rodents at the western location (D54, D55, D123, and D131) were 100% identical to the strain Fyn47, which also originates from the same location. Two bank voles (D97 and D100) from the southeastern location had identical sequences that differ from the sequence of Fyn19. This new sequence was designated as Fyn/97Cg/00 (Fyn97). At the nucleotide level, the difference between western and southeastern strains was 5%. The deduced amino acid sequences of the partial Gc protein were all identical. The complete S segment sequences of the two western strains (Fyn47 and Fyn131) differ from each other by 1.4%, and the southeastern strain Fyn19 differs from these two by 6% (**I**). In a phylogenetic tree based on the partial PUUV M segment sequences, the Danish PUUV strains formed a well-supported lineage (**IV**: Fig. 2). Geographical clustering is further supported as the two strains from southeastern Denmark (Fyn19 and Fyn97) are separated from the western strain (Fyn47). The two bank vole populations, western and southeastern, are separated by agricultural landscape,

and this barrier might slow down gene flow between the two populations. The effective genetic dispersal distance of bank voles has been shown to be no more than 50 km in forested landscapes (Stacy *et al.,* 1997). Our results agree with previous studies, which have shown that in a local rodent population, PUUV strains differ at the nucleotide level up to 2%, and in geographically isolated populations by 6-8% (Plyusnin *et al.,* 1995, Lundkvist *et al.,* 1998, Escutenaire *et al.,* 2001). The two Danish southeastern strains (Fyn19 and Fyn97) were recovered from the same location but 10 years apart, and their difference is 0.8%. Similar results were seen in Montbliart, Belgium, where PUUV strains recovered 10 years apart differed from each other by 0.8-1.1% (Escutenaire *et al.,* 2001).

The hypothesis supported by most studies is that the current PUUV lineages were probably formed after the last ice age during the recolonization of Northern Europe, and they reflect the immigration routes of bank voles (**I**). Based on this hypothesis, we would expect that the closest relative to the Danish PUUV strains would be the southern Scandinavian strains. Our study has provided evidence supporting this hypothesis, but we have also seen that the Danish lineage of PUUV is quite unique. Both the Danish PUUV strains and the Danish bank vole population are also related to the northern lineages (**I**, Jaarola *et al.,* 1999). Thus, we may speculate that PUUV in Denmark represents a recombination between the southern and northern lineages, which has evolved in a rather isolated location. This view is supported by the evidence accumulating for recombination of hantaviruses both *in vivo* and *in vitro* (Sibold *et al.,* 1999, Plyusnin *et al.,* 2002, Chare *et al.,* 2003, **I**, **II**).

Genetic features of the PUUV S segment sequences

The number of recovered PUUV sequences is the largest of all hantaviruses. A collection of 42 complete S segment sequences originating from Europe, Russia and Japan was subjected to a detailed phylogenetic analysis using different methods in an attempt to understand the evolution of this virus. Previous publications on PUUV evolution had only used distance matrix methods on a limited number of sequences originating from specific

geographical areas (Pilaski *et al.*, 1994, Plyusnin *et al.*, 1994, 1995, 1997, 1999, Hörling *et al.*, 1995, Aberle *et al.*, 1999, Heiske *et al.*, 1999, **I**, Escutenaire *et al.*, 2001).

In general, the S segment sequence is a good representative of the whole PUUV genome (**I**), and thus it was chosen for our analysis. The S segment sequences of PUUV vary in length from 1784 nt (strain CG1820) to 1882 nt (strain Sollefteå-6). They all have an ORF for the 433 aa N protein. The 5' NCR is highly conserved, and it is 42 nt in length. The 3' NCR is 442 to 540 nt log, and it is so variable that except for the last ~100 nt, it can only be aligned within a given genetic lineage of PUUV. The overall nt composition of the viral genome is biased: the A content is 33% and the C content only 19%. The transition/transversion ratio is unusually high: 3.5. The mean values of d_S and d_N (the number of synonymous and nonsynonymous substitutions per 100 sites) were calculated for all PUUV sequences, and they were 88±29 and 2.37±0.83. In general, the d_N/d_S ratio was extremely low, suggesting that positive selection is not the primary force driving the evolution of this virus. However, the coding and non-coding regions of the S segment seem to function under different selection pressures and the same might be the case for the different parts of the coding region. For instance, the hypervariable region (aa 233 to 275) of the N protein carries epitopes recognized by both human patient sera and monoclonal antibodies (Lundkvist *et al.*, 1995, Vapalahti *et al.*, 1995). The rate of nonsynonymous substitutions is unusually high within this region, indicating that positive selection may favor certain amino acid replacements in this part of the genome (Hughes and Friedman, 2000). However, even in this region the ratio d_N/d_S does not exceed 1, which would strongly support positive selection (Nei and Gojobori, 1986). Furthermore, the nonsynonymous changes seem random in this region (Hughes and Friedman, 2000, Hughes *et al.*, 1990), suggesting weak functional or structural constraints.

Geographical clustering of PUUV genetic variants

First, a phylogenetic tree of hantavirus N protein sequences was constructed (**II**: Fig. 2). In this tree, hantaviruses form three clades according to their carrier rodents (Murinae, Arvicolinae, and Sigmodontinae rodents). PUUV is found in the Arvicolinae clade

including other viruses carried by voles (Tula, Bloodland lake, Prospect Hill, Isla Vista, and Khabarovsk viruses) and Topografov virus carried by lemmings. Geographical clustering of eight distinct genetic lineages (FIN, RUS, NSCA, SSCA, DAN, BEL, BAL, and JPN) was revealed in a phylogenetic tree based on the PUUV S segment nt sequences (**II**: Fig. 2, Table 1). A common ancestor is shared by the first seven lineages, while the JPN lineage is rather a sister-taxon. The two wild-type strains within the JPN lineage have in fact been recovered from *Myodes rufocanus* trapped in Hokkaido (Kariwa *et al.*, 1995). The Japanese variant also appears to be non-pathogenic for humans (Kariwa *et al.*, 2000). These virus strains should be considered only PUUV-like, since they are carried by a distinct host species. Each of the PUUV genetic lineages is further supported by specific amino acid "signatures" associated with each of them (**II**: Table 1). The most divergent lineage, JPN, has the longest aa signature of 7 specific residues. A closer relationship of the lineages FIN and RUS is indicated as they share two aa markers (Val_{34} and Tyr_{61}). The variation between the different lineages is 15-27% at the nt level, and 0-7.8% at the aa level. Thus, the PUUV N protein diversity sometimes actually exceeds the aa cutoff level of 7%, which is used as one of the criteria to define distinct hantavirus species (Nichol *et al.,* 2005). The intralineage diversity in general is 0.3-9.0% at the nt level. Two lineages, SSCA and RUS, have a higher diversity of 13.4% and 15.6%, and in fact they are both formed by two sublineages.

Star-like phylogeny suggesting an early split of genetic lineages

The overall topology of the PUUV phylogenetic tree is star-like regardless of which method was used in the construction of the tree. The star-like topology suggests an early split of the genetic lineages. The different lineages are well supported, but the relationships between them remain obscure, which further indicates the early split. The bootstrap support values varied depending on the method used, and whether nucleotide or amino acid sequences were used in the calculations (**II**: Table 2). This prompted us to evaluate the accuracy of the tree reconstruction using likelihood mapping option of the TreePuzzle program (Schmidt *et al.*, 2002). This method can be used to visualize the phylogenetic content of a sequence alignment. Only 1.6% of PUUV nucleotide sequence

based quartets were partially or completely unresolved suggesting high accuracy of the tree reconstruction (**II**: Fig. 3). The corresponding value for amino acid sequences was 9.4%, which is higher than for nucleotide sequences, but low enough to consider the reconstruction accurate (Strimmer and von Haeseler, 1997).

Comparison of different phylogenetic methods

One of the aims in this study was to compare different phylogenetic methods, and their performance in analyzing PUUV evolution. We used distance matrix (DM) methods, both Fitch-Margoliash and neighbor joining, and maximum parsimony (MP) method of the PHYLIP package (Felsenstein, 1989). Maximum likelihood (ML) is often considered to be the best choice to infer phylogenies because of the explicit evolutionary model implemented in it. Unfortunately, this method can be very time-consuming, especially with a large data set, and thus traditional ML programs are sometimes inconvenient in practice. This was also true in our case, and we used the program TreePuzzle as a surrogate for ML analysis. TreePuzzle considers only four sequences at a time, thus speeding up the calculations.

All the methods used (DM, MP and ML) agreed on the star-like phylogeny of PUUV. They also assigned strains to their correct phylogeographic genetic lineages, but with variable bootstrap support (**II**: Table 2). As expected, the ML approach gave the most consistent support values, with essentially the same numbers derived with both the nucleotide and amino acid sequences. The bootstrap support values obtained by the MP method were high, especially for the nucleotide sequences, but there was also some inconsistency in the values derived for the nt sequences in comparison to aa sequences. MP works best when the number of sequence changes and, consequently the number of parsimonius sites is relatively small, and apparently the high divergency of PUUV sequences affects the performance of this method. The DM bootstrap support values were the most variable, and in several cases the values were below the generally accepted confidence limit of 70% (Hillis and Bull, 1993). This suggests that the models chosen for correcting distances, Kimura's two-parameter model of nt substitutions or the Dayhoff's

model for aa substitutions, were, to some extent, suboptimal to describe the evolutionary processes in PUUV.

Slow rate of evolution

The assumption of a molecular clock was tested using the ML ratio test (Felsenstein, 1988). The clock was accepted when applying the gamma distribution of rate heterogeneity for nucleotide substitutions (**II**: Fig. 4). The estimated shape parameter alpha was 0.23 ± 0.01 indicating that most of the sites are evolving slowly with few sites having a moderate-to-high rate of evolution. Since the likelihood ratio test was passed, the evolution rate of PUUV could be estimated. Assuming that the viruses have coevolved with their hosts and taking into account the divergence time-points of the carrier rodents, the evolutionary rate based on the number of synonymous substitutions (d_S) was 1.9 x 10^{-7} to 2.2 x 10^{-6} nucleotide substitutions per site per year (**II**: Table3, Fig. 4). The result based on the ML branch lengths of the clock-like trees was very similar, 0.7 x 10^{-7} to 1.1 x 10^{-6} nt substitutions per site per year. Thus all the values were in the range of 0.7 x 10^{-7} to 2.2 x 10^{-6} nt per site per year showing PUUV as a slow evolver. The low value (0.23) of the shape parameter alpha of the gamma distribution is indirectly supporting this slow rate of evolution. The evolution rate of the M segment was estimated to be in the same range (0.37 x 10^{-6} to 0.87 x 10^{-6} nt per site per year) as for the S segment, i.e. both genes of PUUV are apparently evolving at a similar slow rate. This rate is comparable to the evolutionary rates estimated for other stable RNA viruses like human T-cell lymphotropic virus type 2 in tribes infected in endemic regions (Salemi *et al.*, 1999) and hepatitis G virus (Suzuki *et al.*, 1999). Like PUUV, these viruses infect their primary hosts persistently and are well adapted to them. The vast majority of new mutations would probably disturb the equilibrium, in which these viruses remain most of the time close to an adaptive peak, and decrease the fitness of the virus (Salemi *et al.*, 1999).

The rate estimations are based on the current knowledge on the evolution of the rodent hosts. The phylogenies of rodents are based on paleontological records and molecular

data (Catzeflis et al, 1992, Robinson *et al.*, 1997, Kaneko *et al.*, 1998, Conroy and Cook, 1999). The estimated time points for the diversification of rodents are ranging widely in these reports and sometimes they are even controversial. This leads to a somewhat unprecise estimation of the PUUV evolutionary rate. Nevertheless, some events in the PUUV history may now be dated with better accuracy. The split of the Japanese PUUV-like strains and the branch leading to current PUUV strains seems to have happened not later than 100 000 years ago (YA). This could be related to the last Weichselian glaciation of the northern hemisphere starting 115 000 YA. The hypothetical founder populations of the PUUV genetic lineages were then established not later than 85 000 YA. The geographical separation of the present lineages happened during the last deglaciation (21 000 to 17 000 YA). Finally, the immigration routes for flora and fauna to colonize the revealed land after the last ice age are reflected in the evolution of bank voles carrying PUUV (Hewitt, 1999).

Evidence of recombination

Recombination of hantaviruses was first shown for TULV (Sibold *et al.*, 1999, Plyusnin *et al.*, 2002). We have also found evidence for PUUV recombination (**I**), and this mechanism has been suggested also for HTNV (Chare *et al.*, 2003). In general, the rate of homologous recombination in negative-sense RNA viruses seems to be low (Chare *et al.*, 2003). Recombinant sequences were searched for in our collection of complete S segment sequences of PUUV. The pattern of sequence similarities between the different PUUV lineages was first visualized with similarity plots (Salminen *et al.*, 1995, Lole *et al.*, 1999). This analysis suggested that the Baltic strains within the RUS lineage had regions in their sequence, which were actually more similar to the FIN than to the RUS lineage (**II**: Fig. 5). In the molecular clock analysis we had also noticed that the molecular clock was rejected if either of the Baltic strains was included in the phylogenetic tree. This supported the idea of recombinant origin for these sequences, since it has been shown that recombinant sequences disturb the molecular clock (Schierup and Hein, 2000). However, phylogenetic trees created of the partial S segment sequences did not place the Baltic strains within the FIN lineage, but instead they formed a cluster of their own.

Conflicting phylogenetic signals caused by recombination can also be visualized as phylogenetic networks. This analysis was performed with the program SplitsTree, which is based on the split decomposition theory (Bandelt and Dress, 1992). Four lineages (RUS, BEL, NSCA and DAN) were represented as networks in the SplitsTree indicating that these lineages might include recombinant sequences (**II**: Fig. 6). This analysis also provided further evidence for a recombinant origin of the Baltic strains. They were in a cluster of their own at the 5' end and 3' end of the S segment, whereas the middle section of the S segment placed them in the same network with FIN and RUS lineages (**II**: Fig. 6B, 6C, 6D). The networks of BEL, NSCA and DAN lineages are in line with previous results suggesting recombination within those lineages (**I**, Escutenaire *et al.*, 2001).

PUUV evolution in a controlled hantavirus transmission event

Similarly to other RNA viruses, PUUV exists as a population of closely related variants, i.e. quasispecies. Changes in the PUUV quasispecies dynamics were studied in controlled transmission of the virus from one animal to another. The *in vivo* experiments for this study have been describer earlier (Kallio *et al.*, 2006). Colonized bank voles were inoculated with PUU-Kazan wt-strain and kept in individual cages. The cage beddings were contaminated by PUUV excreted from these donor voles. Seventeen days after inoculation the donor voles were removed and recipient voles were placed into the cages where they were exposed to the virus for three days. After the exposure, the recipient voles were moved to individual management cages to develop the infection. Five voles were exposed to each donor consequently. PUUV infection was confirmed using RT-nested-PCR to detect PUUV S segment RNA in lung tissue, and enzyme immunoassay to detect IgG antibodies in serum samples. Bank voles positive for PUUV RNA were used in our study. The region analyzed in this study (nt 631-1630) included both coding region (CR) and 3'-noncoding region (3'-NCR) of the S segment RNA and the selected CR covered both a well-conserved C-terminal and a hypervariable part of the N protein. First, the level of quasispecies diversity was determined. The frequency of mutations in the donor voles injected with the virus was 1.5×10^{-3}, but almost twice as high in the

recipient voles (2.6×10^{-3}) infected with the virus excreted from the donors (**V:** Table 2). The mutation frequency was similar in both coding and non-coding regions of the S segment analyzed and substitutions were evenly distributed across different codon positions. There was a strong bias towards transitions, and there were twice as many nonsynonymous as synonymous substitutions.

An elementary step of PUUV evolution was witnessed in the experiment of controlled virus transmission. One synonymous mutation (A759G) became dominant in the viral quasispecies population during a single virus-transmission event and it was seen in majority of the recipient voles (**V**: Table 3). This apparently advantageous mutation is a synonymous substitution and thus it should affect the virus at the RNA level. Studies on vesicular stomatitis virus have revealed that synonymous mutations may indeed contribute to adaptation of RNA viruses significantly, and this can be accomplished e.g. during different steps of viral replication (Novella *et al.*, 2004). Similarly, the mutation A759G may enhance PUUV replication, since this particular substitution changes the phenylalanine codon to a more abundant one in the host animal. Alternatively the mutation may affect the RNA-protein interactions. A single synonymous substitution was shown to affect the interaction of viral genomic RNA and $Pr55^{gag}$ protein of HIV-1 leading to a dramatic effect on the virion production (Hamano *et al.*, 2007). It remains to be studied, what is the role of this particular region of the hantaviral RNA in interactions with nucleocapsid or some other protein.

Hypophyseal injury caused by Puumala virus

The research on hantavirus pathogenesis has been complicated by the lack of an animal model mimicking human disease and the lack of sensitive methods of virus detection in tissues. Partly this is due to the fact these viruses are highly variable in sequence. We used the knowledge on PUUV divergency to develop a sensitive *in situ* hybridization technique for detection of PUUV RNA in tissues. We also established an immunohistochemical method to detect PUUV antigen in tissue samples. Furthermore, PUUV RNA was detected in clinical samples also by RT-nested-PCR.

The new methods were first evaluated when studying a lethal case of NE. The patient (case 1) was a 58-year-old farmer who was admitted to the hospital and diagnosed with acute NE. The patient died unexpectedly and autopsy samples were examined with immunohistochemical methods. The hypophysis was slightly enlarged with signs of hemorrhage and necrosis (**III**, Fig. 1). There were acute proximal tubular necrosis and small hemorrhages in glomeruli in the kidneys. The liver, spleen, kidneys, and lungs had venous congestion, and the myocardium was fibrotic. PUUV antigen was detected by immunohistochemistry in the hypophysis anterior lobe, kidney tubuli and spleen (**III**, Fig. 2). The infected cells were confirmed as neuroendocrine and endothelial cells in the hypophysis by using cell type specific immunohistochemical staining. PUUV RNA was detected by RT-nested-PCR and *in situ* hybridization in the hypophysis, kidney, lung and spleen. The recovered nt sequence was 78-92% identical to other Puumala virus sequences. This was the first report of Puumala virus detection in the hypophysis.

Altogether three cases of severe NE were described in this study. In addition to the lethal case 1, two other cases were diagnosed by MRI with panhypopituitarism after hypophyseal hemorrhage during or shortly after acute NE. Both of these patients required hormone-replacement therapy. These data suggest that hypophyseal dysfunction following NE might be common and that the burden of possible long-term sequelae or decreased quality of life may be significant.

CONCLUDING REMARKS

This thesis summarizes our studies on the molecular genetics and evolution of Puumala virus (PUUV). In-depth knowledge of the genetic variability and evolution of this human pathogen is essential for understanding NE epidemiology as well as for diagnostics and vaccine development.

In our study, we have shown that PUUV is a genetically diverse hantavirus and that PUUV strains have diverged early on forming distinct geographical lineages. This clustering of PUUV genetic variants reflects the history of bank vole movements during the recolonization of Europe after the last ice age. PUUV evolves mainly through genetic drift, but the data also provide evidence for recombination. This virus is well adapted to its host and the overall evolutionary rate of PUUV is slow. It is, however, a typical RNA virus as it exists as a quasispecies swarm. This quality can accelerate adaptation to a new environment, and in the adaptation process even minor substitutions in the genome may affect the phenotype profoundly. New methods to manipulate and study hantavirus phenotypes need to be developed, and a significant step forward can be taken when the reverse genetics methods are established for hantaviruses.

The constantly increasing sequence data have been useful for the development of new detection methods of PUUV. These have led to an interesting finding of PUUV invading the hypophysis in NE, and the question of prolonged sequelae of the hypophyseal infection has been raised. More studies are clearly needed to understand the involvement of the central nervous system symptoms (CNS) in NE, and the number of cases potentially needing hormone-replacement therapy after the infection. Furthermore, the recent data on NE cases with CNS symptoms have suggested that PUUV genetic variants with severe pathogenicity might circulate in Northern Finland and in Northern Sweden. This emphasizes the importance of studying the currently circulating viral strains and their properties.

Despite many years of work on PUUV and hantavirus pathogenesis, the mechanisms are still poorly understood. The immunohistochemical and *in situ* hybridization methods established in this work will be helpful in studying the monkey model of NE, the first animal model that mimicks the human disease. This will hopefully unravel many of the open questions in hantavirus pathogenesis in near future leading perhaps to new treatment strategies.

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APPENDIX 1

PUUV strains included in specific genetic lineages and studied in this thesis

LINEAGE	PUUV STRAINS	ACCESSION NUMBER (S segment sequences)
FINNISH (FIN)	Puumala	Z46942
	Sotkamo	X61035
	Virrat	Z69985
	Karhumaki	AJ238788
	Kolodozero	AJ238789
	Gomselga	AJ238790
	Evo12	Z30702
	Evo13	Z30703
	Evo14	Z30704
	Evo15	Z30705
	Pallasjarvi63	AJ314597
RUSSIAN (RUS)	Kazan	Z84204
	U338	Z30708
	U444	Z30706
	U458	Z30707
	U894	Z21497
	CG 1820	M32750
	P360	L11347
	Baltic49	AJ314598
	Baltic205	AJ314599
NORTHERN	Tavelsjö	AJ223380
SCANDINAVIAN (N-SCA)	Hundberget	AJ223371
	Mellansel47	AJ223374
	Mellansel49	AJ223375
	Vranica/Hällnäs	U14137
	VindelnL20	Z48586
SOUTHERN	Sollefteå3	AJ223376
SCANDINAVIAN (S-SCA)	Sollefteå6	AJ223376
	Eidsvoll1124v	AJ223368
	Eidsvoll1138	AJ223369
DANISH (DAN)	Fyn131	AJ278093
	Fyn47	AJ278092
	Fyn19	AJ238791
BELGIAN (BEL)	Montbliart23	AJ277031
	Thuin33	AJ277030
	Momignies55	AJ277033
	Momignies47	AJ277032
	Couvin59	AJ277034
	Cg-Erft	AJ238779
	CG 13891	U22423
ALPE-ADRIAN (AA)	Balkan65	AJ314600
	Balkan78	AJ314601
HOKKAIDO VIRUS (HOKV)	Kamiiso	AB010730
	Tobetsu	AB010731