Ecology and evolution of shrew-borne orthohantaviruses in Finland

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Abstract

More than 60% of human emerging infectious diseases (EIDs) are zoonotic (Jones et al., 2008), and they all originate in wildlife. They include infectious diseases, such as Ebola and Middle East Respiratory Syndrome (MERS), and those caused by hantaviruses. Zoonoses are infectious diseases of animals (usually vertebrates) that can be transmitted to humans. Hantaviruses are emerging zoonotic pathogens that belong to the genus *Orthohantavirus* and family *Hantaviridae* in order *Bunyavirales*. Hantaviruses pose a serious threat to human health because their infection causes two highly fatal diseases: haemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS).

Rodents have been regarded as the main reservoir and evolutionary scene of hantaviruses. In the last three decades, our knowledge of hantaviruses has broadened significantly. In contrast to the initial assumption that hantaviruses are mainly carried by rodents, many novel hantaviruses have been detected in shrews, moles and bats during the last few years, and recently, they have even been detected in insects. These findings raise a number of significant questions about the evolutionary history of hantaviruses, their host association and adaptation, the role and frequency of spillover infections and host-switch events, and most importantly, their pathogenicity.

In Finland, Puumala virus (PUUV) has been regarded as the only rodent-borne hantavirus present in the country. It causes a mild form of HFRS called nephropathia epidemica (NE) in humans. To search for novel hantaviruses other than PUUV, various novel hantaviruses were molecularly identified in different species of Soricomorpha ("shrew-

form"). Genetic analyses revealed that four soricomorph-borne hantaviruses circulate in Finland, including Boginia virus (BOGV) in *Neomys fodiens* and Asikkala virus (ASIV) in *Sorex minutus*. Common shrews (*Sorex araneus*) harboured two different hantaviruses: Seewis virus (SWSV) and an Altai-like virus, showing the first evidence of co-existence of two distinct hantavirus species circulating simultaneously in one host species population. This host sharing of two divergent hantaviruses in the European common shrews contradicts hantavirus-host specificity, further implying the complexity of hantavirus evolution.

SWSV and its host *S. araneus* are widespread in Eurasia with a distribution ranging from Central Siberia to Western Europe. After screening hundreds of *S. araneus* trapped from all of Finland, we obtained a large data set of new SWSV sequences that enabled phylogeographic analyses of SWSV. The results demonstrated that this shrew-borne hantavirus is similar to rodent-borne hantaviruses, and the post-glacial spread of SWSV into Finland mirrors that of the host, *S. araneus*: these shrews colonized Finland from the east after the last ice age (12,000–8,000 years ago) and then subsequently spread along emerging land bridges towards the west or north.

The phylogenetic analysis of partial S segment sequences suggested that all Finnish SWSV strains shared their most recent common ancestor with Eastern European strains, whereas the L segment suggested a separate introduction that was most likely via a more northern route. The difference between the L and S segment phylogenies implied that reassortment events played a role in the evolution of SWSV.

From 2001 to 2015, common shrews were collected during several field studies in 45 locations/municipalities across Finland and screened for SWSV. SWSV RNA prevalence in the common shrew population had a spatial but not a temporal pattern with the eastern population having a higher infection rate than the others. SWSV could persistently infect common shrews with some pathological effects. SWSV RNA prevalence in adults or over-wintered common shrews was relatively higher than in summer-born shrews, suggesting that maternal antibodies can protect juvenile shrews from SWSV infection. The exposure risk to SWSV infection increases over the time.

Most new hantaviruses discovered in soricomorph and bat hosts instead of rodents have raised questions as to whether any of them will emerge as human pathogens. Therefore, to predict human exposure risk, novel laboratory techniques for molecular and serological hanta-

virus detection were developed. No evidence of SWSV infection was found among a panel of 486 patient serum samples; however, we demonstrated a cross-reaction of anti-PUUV serum with shrew-borne hantavirus nucleocapsid (N) protein.

This thesis focused on the diversity, host maintenance and cross-species transmission dynamics of soricomorph-borne hantaviruses. The study presented innovative methods to investigate this pertinent topic at the interface of wildlife diseases and human health. The results provided new insights about the ecology, evolutionary origins and phylogeography, and most importantly, the potential pathogenicity of soricomorph-borne hantaviruses. This knowledge in combination with future studies will hopefully lead to a better understanding of host-parasite relationships.

To my family

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Ling J*, Sironen T*, Voutilainen L, Hepojoki S, Niemimaa J, Isoviita V, Vaheri A, Henttonen H, Vapalahti O. Hantaviruses in Finnish soricomorphs: evidence for two distinct hantaviruses carried by *Sorex araneus* suggesting ancient host-switch. *Infect Genet Evol*. 2014, 2;27:51-61.

II Ling J, Smura T, Tamarit D, Huitu O, Voutilainen L, Henttonen H, Vaheri A, Vapalahti O, and Sironen T. Evolution and postglacial colonization of Seewis hantavirus with *Sorex araneus* in Finland. *Infect Genet Evol*. 2018, 57:88-97.

III Ling J, Vaheri A, Hepojoki S, Levanov L, Jääskeläinen A, Henttonen H, Vapalahti O, Sironen T*, Hepojoki J*. Serological survey of Seewis virus antibodies in patients suspected for hantavirus infection in Finland - A cross-reaction between Puumala virus antiserum with Seewis virus N protein? *J Gen Virol*. 2015,96 (7):1664-75.

* Equal contribution

Contents

Abbreviations

aa	amino acid
ASIV	Asikkala virus
BOGV	Boginia virus
BDV	Borna disease virus
CR	chromosomal races
cRNA	complementary RNA
Cyt-B	cytochrome b
dpi	days post infection
DAF	decay-accelerating factor
DEmARC	divErsity partitioning by hierarchical clustering
DMEM	Dulbecco's modified Eagle's medium
ER	endoplasmic reticulum
ESCRT	endosomal sorting complexes required for transport
ELISA	enzyme-linked immunosorbent assay
FRNT	focus reduction neutralization test
FRET	Förster resonance energy transfer
GC1QR	globular heads of complement C1q receptor
GST	glutathione S-transferase
GPC	glycoprotein precursor
GPI	glycosylphosphatidylinositol
HCPS	hantavirus cardiopulmonary syndrome
HFRS	hemorrhagic fever with renal syndrome
HDMEC	human dermal microvascular endothelial cells
HPMEC	human pulmonary microvascular endothelial cells
IB	immunoblotting
IMAC	immobilized metal-ion affinity chromatography
IFA	immunofluorescence assay
IgG	immunoglobulin G
IFN	interferon

ICTV	International Committee on Taxonomy of Viruses
kDa	kilodalton
KHF	Korean hemorrhagic fever
LGM	last glacial maximum
MCMC	markov chain monte carlo
MCC	maximum clade credibility
mRNA	messenger RNA
mAb	monoclonal antibodies
NE	nephropathia epidemica
NGS	next-generation sequencing
nt	nucleotides
PBS	phosphate-buffered saline
POC	point-of-care test
PUUV	Puumala virus
RT-PCR	reverse transcription-polymerase chain reaction assays
RNP	ribonucleoprotein
RdRp	RNA-dependent RNA-polymerase
SWSV	Seewis virus
3D	three-dimensional
TNF-α	tumor necrosis factor alpha
TPMV	Thottapalayam virus
vRNA	viral RNA

1 Introduction

1.1 Hantaviruses and their hosts

Hantaviruses (genus *Orthohantavirus*, family *Hantaviridae,* order *Bunyavirales*) are zoonotic viruses carried by rodents, soricomorphs (e.g. shrews and moles), bats and insects (Guo et al., 2013b; Zhang, 2014). The first hantavirus ever isolated was shrew-associated Thottapalayam virus (TPMV) from an Asian house shrew (*Suncus murinus*) in India in 1971 (Carey et al., 1971). However, its relatedness to hantaviruses was not recognized until many years later. The prototype of rodent-borne hantaviruses is the Hantaan virus (HTNV), which is the causative agent of a disease named Korean haemorrhagic fever (KHF) that was discovered during the Korean war (1951–1953). Ho Wang Lee and Karl M. Johnson identified the virus from a striped field mouse (*Apodemus agrarius*) in 1976 (Lee et al., 1978). Until now, more than 1000 hantavirus strains or lineages have been described and are currently listed in GenBank (http://www.ncbi.nlm.nih.gov). A total of 41 species have been accepted by the International Committee on Taxonomy of Viruses (ICTV) within the genus *Orthohantavirus* (Briese, 2016).

1.1.1 The discovery history of rodent-associated hantaviruses

In Asia, in addition to HTNV, another pathogenic Korean hantavirus was identified and named Seoul virus (SEOV) (Lee et al., 1982a). SEOV is found mainly in rats in China and Southeast Asia, but it has also been found in other locations around the world (Lin et al., 2011) (Table 1 and Figure 1). Meantime, more hantaviruses were identified in Europe. In 1980, the first hantavirus was reported in Finland (Brummer-Korvenkontio et al., 1980). The lung tissue of a bank vole (*Myodes glareolus*) reacted with the sera of Finnish patients with nephropathia epidemica (NE). Immunofluorescence assays revealed a virus related to but distinct from HTNV. This virus, named Puumala virus (PUUV), was isolated by passaging in colonized bank voles

(Brummer-Korvenkontio et al., 1982; Brummer-Korvenkontio et al., 1980). During the Bosnian War (1992–1995), a new hantavirus was isolated from a striped field mouse (*Apodemus flavicollis*) captured in Slovenia and named Dobrava-Belgrade virus (DOBV) (Avsic-Zupanc et al., 1992).

The first hantavirus reported from the Americas was Prospect Hill virus (PHV), which was identified in 1982 (Lee et al., 1982b; Lee et al., 1985). It appears to be non-pathogenic to humans. However, in 1993, a sudden outbreak of a disease with the symptoms of very severe respiratory distress in young, healthy people started to spread in the Four Corner states (New Mexico, Colorado, Utah, and Arizona). Hantavirus pulmonary syndrome (HPS) was first recognized at that time (1993a; 1993b; Centers for Disease and Prevention, 1993; Nichol et al., 1993). The virus was first isolated in 1993 from deer mice (*Peromyscus maniculatus*) and named Sin Nombre virus (SNV) or No Name virus (Hjelle et al., 1994b). After an outbreak of severe pulmonary syndrome among patients in Argentina in 1996, another novel hantavirus was isolated from the long-tailed pygmy rice rat (*Oligoryzomys longicaudatus*) and named Andes virus (ANDV) (Barclay and Rubinstein, 1997). Since then, there have been numerous hantavirus discoveries in South America (mostly partial sequences).

Ever since, more hantaviruses have been found both in Muridae and Cricetidae rodents all over the world, including Africa (Klempa et al., 2012; Witkowski et al., 2014). Even hantaviruses that were thought to be apathogenic to human can cause infections or diseases in humans, such as Tula viruses (Clement and Van Ranst, 2016).

1.1.2 Non-rodent-associated hantaviruses

Shrew or shrew-like mammals are not rodents and more ancient than rodents. They are classified in the order Eulipotyphla or Soricomorpha. The order Soricomorpha (formerly Insectivora) is comprised of four families: Soricidae (shrews), Talpidae (moles), Solenodontidae (solenodons), and Nesophontidae (Nesophontes). Recent genetic analysis suggests that the family Erinaceidae should also be included in the same order as the families of Soricomorpha, and these families together should form the order Eulipotyphla(Beck et al., 2006).

The prototype of soricomorph-borne hantavirus is TPMV, which was isolated from musk shrew during a survey on Japanese encephalitis virus in southern India in 1964 (Carey et al., 1971). TPMV was for decades considered to be the single exception of a hantavirus with a non-rodent reservoir until it was defined based on ultrastructural findings (Zeller et al., 1989). Earlier serological tests showed that it occupies an entirely separate evolutionary lineage (Xiao et al., 1994). It took more than 30 years for this virus to be fully sequenced, and it was regarded as an early divergence from rodent-borne hantaviruses (Song et al., 2007a). Other species of Soricomorpha, including Eurasian common shrew (*Sorex araneus*), Eurasian pygmy shrew (*Sorex minutus*), Eurasian water shrew (*Neomys fodiens*), European mole (*Talpa europaea*), Chinese mole shrew (*Anourosorex squamipes*) and northern short-tailed shrew (*Blarina brevicauda*), have been found presenting HFRS-like antigen in tissue samples or antibodies in early serology studies (Chen, 1986; Gavrilovskaya et al., 1983; Gligic et al., 1992; Lee et al., 1985; Tkachenko et al., 1983). These unspecific findings could not be confirmed until primers were developed (Klempa et al., 2006), allowing the amplification of genome sequences of very distantly related hantaviruses. Since then, many archival tissue samples from shrews, moles, and bats, were screened for the first time for hantaviral RNA. The second shrew-borne virus Tanganya virus (TGNV) was identified in *Crocidura theresae* sampled from Guinea in 2007 (Klempa et al., 2007). Successively, additional hantaviruses were detected in other shrew species: Camp Ripley virus (RPLV) in *Blarina brevicauda* from the USA (Arai et al., 2007), Seewis virus (SWSV) in *Sorex araneus* from Switzerland, Russia and Finland (Song et al., 2007b), and Cao Bang virus (CBNV) in *Anourosorex squamipes* from Vietnam (Song et al., 2007d). These studies revealed the potential of shrews as natural hosts of hantaviruses.

The first mole-borne hantavirus was Asama virus (ASAV), which was identified in *Urotrichus talpoides* (Japanese shrew mole) from Japan (Arai et al., 2008). In 2009, Nova virus was discovered from the European common mole in Hungary (*Talpa europaea*) (Kang et al., 2009c). More interestingly, the mole-borne virus Rockport virus (RKPV), which was identified in the eastern mole (*Scalopus aquaticus*) collected in USA, shared a common ancestor with cricetid-rodent-borne hantaviruses (Kang et al., 2011a). Thus, a new claim that soricomorph-borne hantaviruses have a long-standing evolutionary

history with their hosts started to be accepted by hantavirus researchers.

Bats (Order Chiroptera) are an important animal reservoir that can carry a number of high-impact viral zoonoses, including hantavirus (Olival et al., 2017). In 1994, Kim and colleagues found that bats (*Rhinolophus ferrum-equinum* and *Eptesicus serotinus*) collected in South Korea were serologically positive for HTNV. However, subsequent serological and genetic analyses revealed that the viruses isolated from the bats were HTNV (Kim et al., 1994), which likely indicates contamination. Until 2012, two novel genotypes of hantaviruses were found in bats collected in Western Africa: Magboi virus (MGBV) in *Nycteris hispida* bats in Sierra Leone (Weiss et al., 2012) and Mouyassué virus (MOUV) in *Neoromicia nanus* bats in Côte d'Ivoire (Sumibcay et al., 2012). These bat tissues have been preserved by an ethanol-fixed method and only a partial L segment sequence has been recovered. This partial recovery might also be due to a low viral load in the bat tissues. Later, Xuan Son virus was found in the Pomona roundleaf bat (*Hipposideros pomona*) from Vietnam (Arai et al., 2013). In China, Huangpi virus in the Japanese house bat (*Pipistrellus abramus*) and Longquan virus in three different bats, Chinese horseshoe bat (*Rhinolophus sinicus*), Formosan lesser horseshoe bat (*Rhinolophus monoceros*) and intermediate horseshoe bat (*Rhinolophus affinis*), have been reported (Guo et al., 2013b). Additionally, rodent-borne Araraquara virus was also found in two bat species (*Diphylla ecaudata* and *Anoura caudifer*) in Brazil in 2012 (de Araujo et al., 2012). This finding should be considered with caution. Until now, bat-borne hantaviruses have been found in three families of bats (Rhinolophidae, Nycteridae and Vespertilionidae). Table 2 lists all new hantaviruses species in moles, shrews, and bats worldwide.

1.1.3 Hantaviruses in arthropods

For a long time, hantaviruses were regarded as the only exception to other bunyaviruses, which can all be transmitted by arthropods. Most recently, partial genome sequences of hantaviruses (encoding the L protein) have been found in *Culex* and *Armigeres* mosquitoes (Li et al., 2015), illustrating strong evidence that ancestral non-rodent mammals or insects may have served as the hosts of primordial hantaviruses.

Altogether, more than 80 genetically related viruses have been classified within the *orthohantavirus* family; 25 are recognized as human pathogens responsible for a large spectrum of diseases in the Old and New World. There are more than 90 known reservoirs, belonging to 51 species of rodents, 10 bats (order Chiroptera) and 25 shrews and moles (order Soricomorpha). Figure 1 shows that there are 14 hantaviruses in Soricomorpha and Chiroptera from Asia, 10 from Europe, 8 from Africa, and 6 from North America. Moreover, the diversity of hantaviruses from shrews, moles and bats is higher in Asia than that on other continents, suggesting an Asian origin of extant hantaviruses.

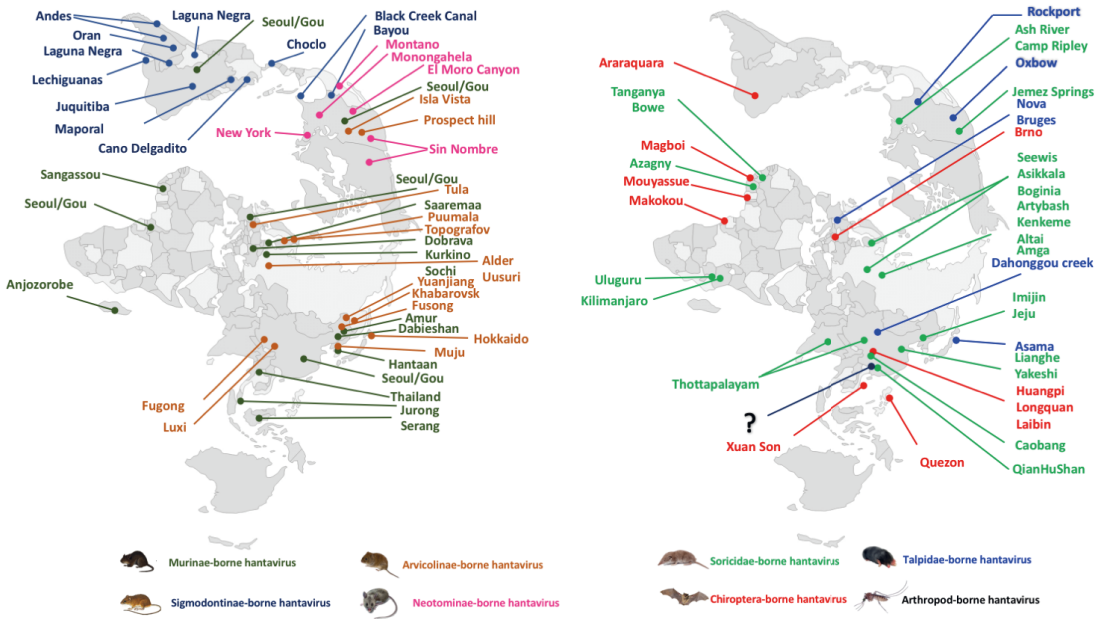

Figure 1. **Landscape of hantaviruses and their host distributions.** Rodent-borne hantaviruses and newly detected non-rodent-borne hantaviruses (viruses are highlighted by the first identified location) indicate an Asian origin and support the emerging concept that ancestral non-rodent mammals or even insects may have served as the hosts of primordial hantaviruses.

Table 1. **List of rodent-borne hantaviruses**

Strains	Species	Potential host	Geographic range	Reference
Hantaviruses carried by Family Muridae, subfamily Murinae				
Amur/ Soochong virus	*Hantaan*	*Apodemus peninsulae* (Korean field mouse)	E Asia	(Liang et al., 1994; Yashina et al., 2001)
Hantaan virus (HTNV)	*Hantaan*	*Apodemus agrarius* (striped field mouse)	E Eurasia	(Lee et al., 1982a)
Da Bie Shan virus (DBSV)	*Dabieshan*	*Niviventer confucianus* (Chinese white-bellied rat)	E Asia	(Wang et al., 2000)
Dobrava-Belgrade virus (DOBV)	*Dobrava-Belgrade*	*Apodemus flavicollis* (yellow-necked mouse)	Europe	(Avsic-Zupanc et al., 1992)
Saaremaa virus (SAAV)	*Dobrava-Belgrade*	*Apodemus agrarius* (striped field mouse)	Estonia (Saaremaa Island)	(Nemirov et al., 1999)
Kurkino virus	*Dobrava-Belgrade*	*Apodemus agrarius* (striped field mouse)	Russia	(Plyusnin et al., 1999)
Sochi virus	*Dobrava-Belgrade*	*Apodemus ponticus* (Black sea field mouse)	Russia	(Klempa et al., 2008)
Sangassou virus (SANGV)	*Sangassou*	*Hylomyscus simus* (African wood mouse)	Côte d'Ivoire, Africa	(Klempa et al., 2006)
Seoul virus (SEOV)	*Seoul*	*Rattus rattus* (black rat) *Rattus norvegicus* (brown rat)	Global	(Elwell et al., 1985)
Gou virus (GOUV)	*Seoul orthohantavirus*	*Rattus rattus* (black rat)	Global	(Wang et al., 2000)

Serang virus		*Rattus tanezumi* (Asian house rat)	Indonesia	(Plyusnina et al., 2009)
Thailand virus (THAIV)	*Thailand*	*Bandicota indica* (great bandicoot rat)	S Asia	(Xiao et al., 1994)
Anjozorobe virus	*Thailand*	*Rattus rattus* (ubiquist roof rat) *Eliurus majori* (Major's tufted-tailed rat)	Madagascar	(Reynes et al., 2014)
Jurong virus	*Thailand*	*Rattus tanezumi* (Asian house rat)	Singapore	(Johansson et al., 2010)
Hantaviruses carried by Family Cricetidae, subfamily Arvicolinae				
Fugong virus	*Fugong*	*Eothenomys eleusis* (Small oriental vole)	China	(Ge et al., 2016)
Fusong virus	*Fusong*	*Microtus fortis* (Reed vole)	China	(Zou et al., 2008b)
Isla Vista virus (ISLAV)		*Microtus californicus* (Californian vole)	North America	(Song et al., 1995)
Khabarovsk virus (KHAV)	*Khabarovsk*	*Microtus maximowiczii* (Maximowicz's vole)	Far-eastern Russia	(Horling et al., 1996)
Topografov (TOPV)	*Khabarovsk*	*Lemmus sibiricus* (Siberian brown lemming)	Palaearctic tundra	(Vapalahti et al., 1999)
Luxi virus (LUXV)	*Luxi*	*Eothenomys miletus* (Yunnan red-backed vole)	China	(Zhang et al., 2011)
Prospect Hill virus (PHV)	*Prospect*	*Microtus pennsylvanicus* (meadow vole)	North America	(Lee et al., 1985)
Puumala virus (PUUV)	*Puumala*	*Myodes glareolus* (bank vole)	Europe to W Siberia	(Brummer-Korvenkontio et al., 1980)

Hokkaido virus (HOKV)	*Puumala*	*Myodes rufocanus* (grey-sided vole)	Eurasia	(Kariwa et al., 1999)
Muju (MUJV)	*Puumala*	*Myodes regulus* (royal vole)	Korea	(Song et al., 2007e)
Tula (TULV)	*Tula*	*Microtus arvalis* (European common vole)	Eurasia	(Plyusnin et al., 1994)
Alder virus (ADLV)	*Tula*	*Microtus majori* (Major's pine vole)	Russia	(Tkachenko et al., 2015)
Ussuri virus		*Myodes rufocanus*	Russia	
Yuanjiang (YUJV)		*Microtus fortis* (reed vole)	China	(Zou et al., 2008c)
Hantaviruses carried by Family Cricetidae, subfamily Neotominae				
El Moro canyon virus (ELMCV)	*El Moro canyon*	*Reithrodontomys megalotis* (western harvest mouse)	North America	(Hjelle et al., 1994a)
Carrizal virus (CARV)	*El Moro canyon*	*Reithrodontomys sumichrasti* (Sumichrast's harvest mouse)	Mexico	(Kariwa et al., 2012)
Huizilac virus (HUIV)	*El Moro canyon*	*Reithrodontomys mexicanus* (Mexican harvest mouse)	Mexico	(Kariwa et al., 2012)
Limestone Canyon (LSCV)		*Reithrodontomys mexicanus* (Mexican harvest mouse)	North America	(Sanchez et al., 2001)
Monongahela		*Peromyscus maniculatus* (deer mouse)	North America	(Rhodes et al., 2000)
Montano virus (MTNV)	*Montano*	*Peromyscus beatae* (Orizaba deermouse)	Mexico	(Kariwa et al., 2012)
Rio Segundo virus (RIOSV)		*Reithrodontomys mexicanus* (Mexican harvestmouse)	Mexico	(Hjelle et al., 1995a)

Sin Nombre (SNV)	*Sin Nombre*	*Peromyscus maniculatus* (deer mouse)	North America	(Nichol et al., 1993)
New York (NYV)	*Sin Nombre*	*Peromyscus leucopus* (white-footed mouse)	North America	(Hjelle et al., 1995b)
Hantaviruses carried by Family Cricetidae, subfamily Sigmodontinae				
Alto Paraguay virus (ALPV)		*Holochilus chacarius* (Chacoan marsh rat)	Paraguay	(Chu et al., 2003)
Anajatuba (ANAJV)		*Oligoryzomys fornesi* (Fornes' colilargo)	Brazil	(Rosa et al., 2005)
Andes virus (ANDV)	*Andes*	*Oligoryzomys longicaudatus* (long-tailed pigmy rice rat)	Argentina	(Lopez et al., 1996)
Lechiguanas virus (LECV)	*Andes*	*Oligoryzomys flavescens* (yellow pygmy rice rat)	Argentina	(Levis et al., 1997)
Oran virus (ORNV)	*Andes*	*Oligoryzomys longicaudatus* (long-tailed pygmy rice rat)	Argentina	(Bohlman et al., 2002)
Castelo dos sonhos (CASV)	*Andes*	*Oligoryzomys eliurus* (Brazilian pygmy rice rat)	Brazil	(Johnson et al., 1999)
Cano Delgadito virus (CADV)	*Cano Delgadito*	*Sigmodon alstoni* (cane mouse)	South America	(Fulhorst et al., 1997)
Araraquara (ARAV)		*Bolomys lasiurus* (hairy-tailed bolo mouse)	Brazil	(Johnson et al., 1999)
Araucaria (ARAUV)		*Oligoryzomys nigripes* (black-footed pygmy rice rat) *Oxymycterus judex* (hocicudo) *Akodon montensis* (montane grass mouse)	Brazil	(Rosa et al., 2005)

Bayou virus (BAYV)	*Bayou*	*Oryzomys palustris* (rice rat)	North America	(Morzunov et al., 1998)
Catacamas (CATV)	*Bayou*	*Oryzomys couesi* (Coues' rice rat)	Honduras	(Milazzo et al., 2006)
Black Creek Canal virus (BCCV)	*Black Creek Canal*	*Sigmodon hispidus* (hispid cotton rat)	North America	(Ravkov et al., 1995)
Choclo (CHOV)	*Choclo*	*Oligoryzomys costaricensis* (Costa Rican pygmy rice rat)	Panama	(Vincent et al., 2000)
Itapua (ITPV)		*Oligoryzomys nigripes* (black-footed pygmy rice rat)	Paraguay	(Chu et al., 2003)
Jabora (JABV)		*Akodon montensis* (montane grass mouse)	Paraguay	(Goodin et al., 2009)
Jupuitiba (JUQV)		*Oligoryzomys fornesi* (Fornes' colilargo)	Brazil	(Vasconcelos et al., 1997)
Laguna Negra virus (LANV)	*Laguna Negra*	*Calomys laucha* (vesper mouse)	Paraguay and Bolivia	(Johnson et al., 1997)
Maripa virus (MCLV)	*Laguna Negra*	*Bolomys obscurus* (dark bolo mouse)	Argentina	(Levis et al., 1997)
Rio Mamore virus (RIOMV)	*Laguna Negra*	*Oligoryzomys microtis* (small-eared pygmy rice rat)	Bolivia	(Hjelle et al., 1995b)
Maciel (MCLV)		*Bolomys obscurus* (dark bolo mouse)	Argentina	(Levis et al., 1997)
Maporal virus (MAPV)	*Maporal*	*Oligoryzomys delicatus* (delicate pygmy rice rat)	West Venezuela	(Fulhorst et al., 2004)
Muleshoe (MULV)		*Sigmodon hispidus* (hispid cotton rat)	North America	(Rawlings et al., 1996)

Pergamino (PRGV)		*Akodon azarae* (Azara's grass mouse)	Argentina	(Bohlman et al., 2002)
Playa de Oro (OROV)		*Sigmodon mascotensis* (Jaliscan cotton rat) *Oryzomys couesi* (Coues' rice rat)	Mexico	(Chu et al., 2008)
Rio Mearim (RIMEV)		*Holochilus sciureus* (Amazonian marsh rat)	Brazil	(Rosa et al., 2005)
Hantaviruses carried by Order Rodentia, Family Thryonomyidae				
Necoclí Virus (NECV)	*Necocli*	*Zygodontomys cherriei* (Cherrie's cane rat)		(Londono et al., 2011)

Table 2. **List of nonrodent-borne hantaviruses**

Strains	Species	Potential host	Place first found	Reference
Hantaviruses carried by Order Eulipotyphla, Family Soricidae				
Altai virus (ALTV)		*Sorex araneus* (Eurasian common shrew)	Russia	
Artybash virus (ARTV)	*Amga*	*Sorex caecutiens* (Laxmann's Shrew)	Russia	(Arai et al., 2016)
Asikkala virus (ASIV)	*Asikkala*	*Sorex minutus* (Eurasian pygmy shrew)	Europe	(Radosa et al., 2013b)

Ash River virus		*Sorex cinereus* (masked shrew)	Minnesota	(Arai et al., 2008a)
Azagny (AZGV)		*Crocidura obscurior* (West African pygmy shrew)	Côte d'Ivoire	(Kang et al., 2011b)
Bowé virus (BOWV)	*Bowé*	*Crocidura theresae* (Therese's shrew)	Guinea	(Gu et al., 2013b)
Camp Ripley virus (RPLV)		*Blarina brevicauda* (northern short-tailed shrews)	United States	(Arai, Song et al. 2007)
Cao Bang virus (CBNV)	*Cao Bang*	*Anourosorex squamipes* (Chinese mole shrew)	Vietnam	(Song et al., 2007d)
Lianghe virus (LHEV)	*Cao Bang*	*Anourosorex squamipes* (Chinese mole shrew)	China	(Guo et al., 2013b)
Imjin virus (MJNV)	*Imjin*	*Crocidura lasiura* (Ussuri white-toothed shrew)	Republic of Korea	(Song, Kang et al. 2009)
Jeju virus (JJUV)	*Jeju*	*Crocidura shantungensis* (Asian lesser white-toothed shrew)	Republic of Korea	(Arai et al., 2012)
Jemez Springs virus		*Sorex monticolus* (dusky shrew)	New Mexico and Colorado	(Arai et al., 2008a)
Kenkeme virus (KKMV)	*Kenkeme*	*Sorex roboratus* (flat-skulled shrew)	Sakha Republic in northeastern Siberia	(Kang et al., 2010)
Kilimanjaro (KMJV)		*Myosorex zinki* (Kilimanjaro mouse shrews)	Tanzania	(Kang et al., 2014)
Qian Hu Shan virus (QHSV)		*Sorex cylindricauda* (stripe-backed shrew)	China	(Zuo et al., 2014)
Seewis virus (SWSV)		*Sorex araneus* (Eurasian common shrew)	Graubünden, Switzerland	(Song et al., 2007c)

Tanganya (TANGV)		*Crocidura theresae* (Therese's shrew)	Guinea, West Africa	(Klempa et al., 2007)
Thottapalayam (TPMV)	*Thottapalayam*	*Suncus murinus* (Asian house shrew)	India	(Carey et al., 1971)
Uluguru virus		*Myosorex geata* (Geata mouse shrew)	Tanzania	(Kang et al., 2014)
Xinyi virus (XYIV)		*Anourosorex yamashinai* (Taiwanese mole shrew)	China	(Gu et al., 2016a)
Yakeshi	*Yakeshi*	*Sorex isodon*	China	(Guo et al., 2013b)
Hantaviruses carried by Order Eulipotyphla, Family Talpidae				
Asama virus (ASAV)	*Asama*	*Urotrichus talpoides* (Japanese shrew mole)	Japan	(Arai et al., 2008b)
Bruges virus (BRGV)	*Bruges*	*Talpa europaea* (European common mole)		
Dahonggou Creek (DHCV)		*Scaptonyx fusicaudus* (a long-tailed mole)	China	(Kang et al., 2016)
Nova (NVAV)	*Nova*	*Talpa europaea* (European common mole)	Hungary	(Kang et al., 2009c)
Oxbow (OXBV)	*Oxbow*	*Neurotrichus gibbsii* (American shrew mole)	Gresham, Oregon	(Kang et al., 2009b)
Rockport (RKPV)	*Rockport*	*Scalopus aquaticus* (eastern mole)	United state	(Kang et al., 2011a)
Hantaviruses carried by Order Chiroptera				

Araquare-like virus		*Diphylla ecaudata* and *Anoura caudifer*	Brazil	(de Araujo J et al. 2013)
Brno (BRNV)		*Nyctalus noctula*	Czech Republic	
Huangpi virus		*Pipistrellus abramus* (Japanese house bat)	China	(Guo et al., 2013b)
Laibin virus (LBV)	*Laibin*	*Taphozous melanopogon* (black-bearded tomb bat)		(Xu et al., 2015)
Longquan virus	*Longquan*	*Rhinolophus affinis*, *Rhinolophus sinicus*, and *Rhinolophus monoceros*	China	(Guo et al., 2013b)
Magboi virus (MGBV)		*Nycteris hispida* (Hairy split-faced bat)	Magboi River in Sierra Leone	(Weiss et al., 2012)
Makokou virus (MAKV)		*Hipposideros ruber* (Noack's roundleaf bat)		(Witkowski et al., 2016)
Mouyassué virus (MOUV)		*Neoromicia nanus* (banana pipistrelle)	Côte d'Ivoire, West Africa	(Sumibcay et al., 2012)
Quezon virus (QZNV)	*Quezon*	*Rousettus amplexicaudatus* (Geoffroy's rousette)	Philippines	(Arai et al., 2016)
Xuan Son virus (XSV)		*Hipposideros pomona* (Pomona roundleaf bat)		(Arai et al., 2013)

Hantavirus taxonomy according to (Briese, 2016).The official hantavirus species is given in italics; other tentative virus species are a)Altai virus, b) Camp Ripley virus, c) Isla Vista virus, d) Jemez Springs virus, e) Kilimanjaro virus, f) Muleshoe virus, g) Qian Hu Shan virus, h) Rio Segundo virus, i) Seewis virus, j) Shenyang virus, k) Serang virus, l) Uluguru virus, m) Ussuri virus, n) Xinyi virus, o) Xuan Son virus, and p)Yuanjiang virus.

1.2 Hantavirus classification

The discovery of novel genotypes of hantaviruses from bats and insectivores over the past 10 years has promoted an update of hantavirus classifications. Meanwhile, increasing numbers of bunyaviruses have been discovered in animals, plants, humans, and arthropods. The International Committee on Taxonomy of Viruses (ICTV) proposed in 2016 that the old classification (family *Bunyaviridae*) must be elevated into a new order (*Bunyavirales*) to accommodate nine families (eight new, one renamed: *1. Feraviridae 2. Fimoviridae 3. Hantaviridae 4. Jonviridae 5. Nairoviridae 6. Phasmaviridae 7. Phenuiviridae* and *8. Tospoviridae*), comprising thirteen genera (Briese, 2016). This classification is substantiated mainly by phylogenetic analyses, including diversity partitioning by hierarchical clustering (DEmARC) analysis and Bayesian Markov chain Monte Carlo (MCMC) phylogeny using concatenated S, M, and L segment sequences of bunyaviruses (Briese, 2016). As the ICTV does not offer ranks, such as subgenus or subspecies, for hantaviruses, an elevation of the genus (*Ortho*)*hantavirus* to family (*Hantaviridae*) creates the necessary room to create subfamilies and additional genera in the future. Currently, 41 species and 16 tentative members in the genus (*Ortho*)*hantavirus* have been approved in the new classification, and this approval solely relies on genetic data. As a result, New York virus (NYV), Rio Mamoré virus (RIOMV), Saaremaa virus (SAAV), and Topografov virus (TOPV) have been regarded as strains and regrouped into the species *Sin Nombre orthohantavirus*, *Laguna Negra orthohantavirus*, *Dobrava-Belgrade orthohantavirus*, and *Khabarovsk orthohantavirus*, respectively. However, this rule is still under debate. Regarding hantavirus nomenclature, sequence data are not the only factor that should be taken into consideration. In this case, the old classification by the ICTV seems more accurate since more virological aspects are taken into consideration; a hantavirus species should have (i) been found in a unique ecological niche, i.e., in a specific primary reservoir species or subspecies, (ii) at least 7% amino acid (aa) divergence in the complete nucleocapsid (N) and glycoprotein precursor (GPC) proteins, (iii) at least a 4-fold difference in a two-way cross neutralization test, and (iv) no naturally occurring reassortants (Plyusnin, 2011).

1.3 Hantavirus structure and genome organization

1.3.1 Schematic structure of hantavirus virions

Hantaviruses form enveloped, spherical virions of 80–160 nm in size. By mass, the virion is greater than 50% protein, 20–30% lipid and 2–7% carbohydrate. The density of the virions is 1.18 gram per cubic centimetre (Schmaljohn et al., 1985). They can remain infectious for more than 10 days at room temperature and more than 18 days at +4 °C and at −20 °C (Kallio et al., 2006a). This feature is important for hantavirus transmission.

The virions are enveloped and contain a single-stranded, negative-sense, tri-segmented RNA genome, which consists of small (S), medium (M) and large (L) segments, encoding nucleocapsid protein (N), the glycoprotein precursor (GPC) of two glycoproteins (Gn and Gc), and RNA-dependent RNA polymerase (RdRp), respectively. Within the virions, the genomic RNA of hantaviruses is thought to complex with N protein to form helical nucleocapsids, the RNA component of which circularizes and forms a ‘panhandle’ structure due to sequence complementarity between the 5' and 3' terminal sequences (AUCAUCAUC) of the genomic segments, which is complementary to the 5' terminal (Schmaljohn, 2007; Spiropoulou, 2011).

The length of the S segment is 1696–2083 nucleotides (nt), the M segment is 3613–3707 nt and the L segment is 6530–6550 nt. At the 5' and 3' ends of each segment are short noncoding sequences. The noncoding segment in all sequences at the 5' end is 37–51 nt. The 3' noncoding regions differ: it ranges from 370–730 nt in the S segment; 168–229 nt in the M segment; and 38–43 nt in the L segment (Vaheri et al., 2013b).

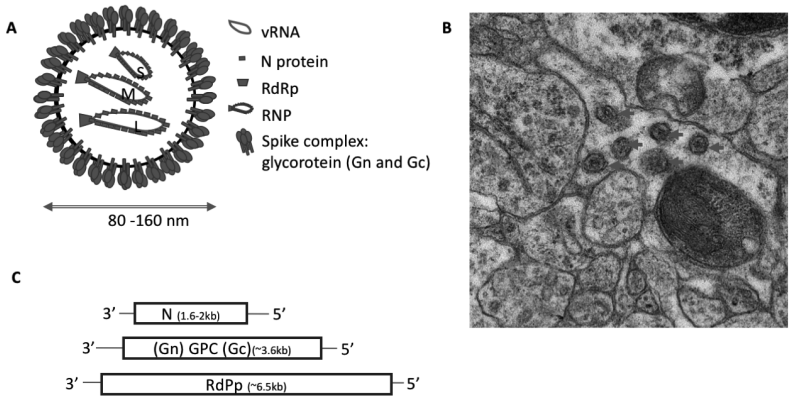

Figure 2. (A) Hantaviral structure. The lipid bilayer of the viral envelope is about five nm thick and is embedded with viral surface proteins; the glycoproteins, known as Gn and Gc, are encoded by the M segment. They are heterodimerized with each other and have an interior tail and an exterior domain. Nucleocapsids are inside the envelope, and they interact with the three segments of the viral genome to form helical structures. RNA polymerase is also found in the interior. (B) Electron micrograph (EM) of Hantaan virus virions in the brain tissue of BALB/c mouse (Li et al., 2013). The arrowheads show virus particles, whose diameter ranged from 91.8 to 117.82 nm. (C) Genome organization of a hantavirus.

1.3.2 Proteins of hantavirus

Nucleocapsid protein

N protein is a non-glycosylated protein with a molecular mass of approximately 50 kDa (429–433 aa) (Hussein et al., 2011; Kaukinen et al., 2005). The predicted secondary structure showed that the first 75 aa is a coiled-coil domain, and it has been confirmed by nuclear magnetic resonance (NMR) for ANDV N protein (Alfadhli et al., 2002; Wang et al., 2008). The core part of N protein (175–218 aa) is a RNA-binding region, which was also confirmed with a crystal structure (Guo et al., 2015; Olal and Daumke, 2016). N protein is the major structural component of the virus with multiple functions. It exists as a trimer, which is essential for hantavirus replication (Kaukinen et al., 2004; Mir and Panganiban, 2004). Studies revealed that hantavirus N protein simultaneously binds both m^7G caps and viral RNA (vRNA) at distinct sites, suggesting that hantavirus N protein has important func-

tions in cap snatching and viral mRNA synthesis in the processing (P) body (Flick et al., 2003; Mir et al., 2008; Patterson and Kolakofsky, 1984). In addition, N protein can interact with other viral structural proteins (Cheng et al., 2014). N protein can also interact with cellular proteins, including Daxx and the SUMO-1 pathway, and with actin filaments and microtubules (Kaukinen et al., 2003; Lee et al., 2003). N protein is a target for host innate immunity against infection via its association with MxA-mediated antiviral response (Kanerva et al., 1996). It also has been found to interfere with host innate immune responses by downregulating apoptosis (Park et al., 2013), inhibiting interferon (IFN) signalling responses (Cimica et al., 2014; Levine et al., 2010), and blocking tumour necrosis factor alpha (TNF-α)-induced activation of NF-κB (Taylor et al., 2009) .

N protein has an immunodominant antigenic region that is composed of linear epitopes near the first 100 aa of the N-terminus. The region is antigenically cross-reactive among viruses in the same group but not to viruses in different groups. However, a serotype-specific and multimerization-dependent antigenic site (conformation-dependent epitopes) was found in the C-terminal half of N protein (Yoshimatsu and Arikawa, 2014a).

Glycoproteins (Gn and Gc)

The glycoprotein precursor encoded by the M segment is cleaved directly after translation at the conserved WAASA motif into two glycoproteins, Gn (~70 kDa) and Gc (~55 kDa). For further maturation of hantavirus glycoprotein, N-linked glycosylation is essential. Four glycosylation sites have been identified on HTNV Gn (N134, N235, N347 and N399) and one on HTNV Gc (N928) (Shi and Elliott, 2004). Three-dimensional (3D) structures of PUUV, TULV and HTNV spike complexes, derived by electron cryo-microscopy studies and combined with biochemical analysis, revealed that Gn and Gc form square-shaped surface spikes of four-fold symmetry on the virion envelope (Battisti et al., 2011; Hepojoki et al., 2010; Huiskonen et al., 2010; Li et al., 2016). Gn and Gc play major roles in viral attachment and entry to the host cells, virulence, and assembly and packaging of new virions in infected cells. The Gn cytoplasmic tail also contains a late domain motif (YXXL) that in other viruses has been shown to interact with cellular factors that facilitate virus budding from cells. Gn-CT can mediate the downregulation of Gn through

ubiquitination (Wang et al., 2009). It may function as a late domain for the recruitment of cellular Endosomal Sorting Complexes Required for Transport (ESCRT) complexes (Strandin et al., 2013). Gc in hantaviruses is a class II membrane fusion protein (Guardado-Calvo et al., 2016). A recent report described for the first time that the Gn and Gc glycoproteins of ANDV and PUUV self-assemble into virus-like particles (VLPs) (Acuna et al., 2014).

RdRp

The largest protein encoded by hantaviruses is RdRp with a molecular size of approximately 250 kDa (Kukkonen et al., 2004; Spiropoulou, 2011). L RNA encodes L protein, and at least five motifs of its amino acid (aa) sequence are highly conserved, which is similar to the other polymerases of negative stranded viruses; the conserved sites are found at aa positions 884–902, 964–980, 1050–1077, 1091–1101, 1152–1164 and 1171–1181, including an XDD motif that is essential for catalytic activity (Kukkonen et al., 2005). RdRp functions as a RNA replicase and transcriptase to replicate and transcribe viral RNA and is also thought to have endonuclease activity (Jonsson and Schmaljohn, 2001; Reguera et al., 2010; Rothenberger et al., 2016). RdRp has no proofreading/repair mechanism or post-replicative error correction mechanism. More detailed information about the molecular or biochemical characteristics of RdRp are very limited because no efficient heterologous expression of RdRp has been obtained and no suitable reverse genetic system has been established for hantaviruses so far.

Non-structural protein

The S segment of hantaviruses carried by Cricetidae rodents contains an overlapping reading frame that encodes the non-structural protein NSs, which can function as a weak IFN inhibitor as in the other viruses of *Bunyavirales* (Jaaskelainen et al., 2007; Ronnberg et al., 2012; van Knippenberg et al., 2013). Moreover, NSs is likely to have other functions, including RNA silencing (Hedil and Kormelink, 2016; Ronnberg et al., 2012; Virtanen et al., 2010).

1.3.3 Replication cycle

Infection starts by the binding of hantavirus Gn and Gc to integrin receptors of the human cell: β1 integrin (the ligand fibronectin) is im-

plicated in the infection of non-pathogenic hantaviruses (including TPMV) (Song et al., 2007a), whereas β3 integrin (the ligand vitronectin) is implicated for pathogenic species (Gavrilovskaya et al., 1998; Mackow and Gavrilovskaya, 2001). Decay-accelerating factor (DAF), a glycosylphosphatidylinositol (GPI)-anchored protein of the compelement system, has been suggested as a critical co-factor for entry (Krautkramer and Zeier, 2008). Globular heads of the complement C1q receptor (GC1QR) can also mediate hantavirus infection in cultured cells (Choi et al., 2008). After binding, virus entry occurs via clathrin-mediated endocytosis. The endo-lysosome and viral membrane are fused, and the virus is decapsulated in endolysosomal compartments to liberate the viral genome (Jin et al., 2002). The ribonucleoproteins (RNPs) are transported to the perinuclear region, where RdRp immediately starts the transcription of messenger RNA (mRNA). The primers needed for viral transcription are acquired from host cell mRNAs through a process called cap snatching (Bouloy et al., 1978) in which 5'-capped oligonucleotides (10–14 nt long) are generated by endonuclease encoded by the L segment. Degraded cellular mRNAs accumulate in cytoplasmic processing bodies (P bodies). Soon after initial transcription, the synthesis of antigenomic complementary RNA (cRNA) starts. N protein in the trimeric conformation binds the viral RNA (vRNA) panhandle, which starts to unwind but remains attached to the 5' terminus, leaving the 3' terminus accessible to L protein (Mir and Panganiban, 2006). The initiation of both transcription and replication has been suggested to follow the "prime-and-realign" mechanism (Garcin et al., 1995). The produced cRNA will in turn be used as a template for new vRNA production. The produced vRNA can serve as an additional template for mRNA or as the genome of progeny virions.

The translation of viral S, M and L mRNA takes place on ribosomes, similar to the translation of any other cellular protein. L and S segment mRNA is translated on free ribosomes to generate RdRp and N, respectively. N protein is the first protein to be synthesized and accumulates immediately following infection to protect the vRNA from degradation (Severson et al., 2001).

M segment mRNA is translated on membrane-bound ribosomes to generate GPC, which is cleaved to Gn and Gc at the conserved WAASA motif. After the addition of N-linked oligosaccharides, the Gn and Gc proteins dimerize in the endoplasmic reticulum (ER)

(Lober et al., 2001). The interaction of Gn and Gc in the ER seems to be crucial in order for them to be transported to the Golgi complex (Ruusala et al., 1992). Glycoproteins are transported in vesicles through yet unknown mechanisms to the Golgi, where they accumulate (Pensiero et al., 1988). Retention of the glycoproteins at the Golgi complex is thought to be responsible for the maturation of hantaviruses in the Golgi, where assembly takes place. Unlike many other negative stranded RNA viruses, hantaviruses do not have a matrix protein. Therefore, it is likely that the cytoplasmic tails of Gn and Gc interact directly with N during assembly. In contrast to Golgi maturation, EM studies of SNV and BCCV have suggested the cell surface as an alternative maturation and budding site for New World hantaviruses.

1.4 Enzoonotic cycle of hantaviruses

Hantaviruses are transmitted between rodent species either directly via contacts or indirectly via the environment (Botten et al., 2002; Kallio et al., 2006a). The transmission route of non-rodent-associated hantaviruses is still unknown. Soricomorphs and rodents share the same ecological niche, and some of their physical characteristics are quite similar; for instance, they both have small body sizes, but shrews, moles and bats belong to a different taxonomic order, and the main ecological features of soricomorphs, such as metabolism, age at maturity, litter size and maximum lifespan, are distinct from rodents. Current knowledge on the ecology of non-rodent-borne hantaviruses is scarce. Nevertheless, we can extrapolate based on the knowledge that we have acquired from rodent-borne hantaviruses. Rodent-borne hantavirus infection induces a life-long antibody response in rodent species approximately 2–3 weeks post-infection, and rodents can be persistently infected without experiencing any detrimental effects and can maintain significant levels of virus that can be shed or transmitted (Korva et al., 2009; Kuenzi et al., 2005; Voutilainen et al., 2015). For shrew-borne hantavirus, detectable hantaviral RNA existed among over-wintered shrews, suggesting that shrews can be persistently infected with shrew-borne hantaviruses (our unpublished data). Many factors, including viral, host or environmental ones, can influence transmission between hosts.

1.4.1 Hantavirus maintenance in the host

Rodent-borne hantaviruses can be found in the blood and many other organs, such as the kidneys, liver, spleen, heart, and most abundantly, in the lungs (Lee et al., 1981; Spengler et al., 2013; Yanagihara et al., 1984). The virus is also found in the saliva, urine and faeces of rodents (Hardestam et al., 2008). Shrew-borne hantaviruses have been recovered from lungs, kidney, livers and also spleens (Lee et al., 2017). Bat-borne hantavirus can be found in gut and brain and are distributed predominantly in the spleen and kidney (Witkowski et al., 2016).

Host behaviour can to a large extent influence the transmission rate of a pathogen. A more aggressive behaviour, like fighting of the host, could benefit the transmission of a hantavirus (Hinson et al., 2004). Male adult rodents have a higher risk of hantavirus infection that is related to aggressive behaviour; examples include SEOV in rats, PUUV in bank voles and SNV in deer mice. (Deter et al., 2008; Klein et al., 2004; Olsson et al., 2002). The same result was also found in the shrew-borne hantavirus Imjin virus (Lee et al., 2017). The mechanisms could be hormone-related since hantaviruses have been shown to infect the testes (Hinson et al., 2004). Hormones might either change male behaviour, and thereby increase their exposure to the virus, or hormones regulate the immune response, thereby rendering the males susceptible to infection. In addition, maternal antibodies also contribute to sex-based differences in hantavirus transmission dynamics (Kallio et al., 2013). The overall seropositivity among rodents varies by area and species, ranging from up to 9.5–11% in deer mouse and 15–50% in bank voles.

In wild captured rodents, weight (used as an indicator of age) strongly correlates with the prevalence of hantavirus infection, which indicates a horizontal transmission route (Meyer and Schmaljohn, 2000; Voutilainen et al., 2012). It has been shown that PUUV infected bank voles have a lower over-winter survival probability than antibody-negative bank voles (Kallio et al., 2007). However, another study showed that PUUV-seropositive voles had a higher survival probability from spring to summer. These results indicate that PUUV infection might have effects on host fitness (Reil et al., 2017). The increased seroprevalence observed among young animals is explained by the transfer of antibodies from the mother to pups via the placenta and during lactation (Dohmae et al., 1993). Passive immunization with maternal anti-

bodies has been reported to protect the pups for 3–3.5 months or up to 145 days (Kallio et al., 2006b). This passive immunization is also the reason for the similar prevalence of hantavirus infection in both increased and peak years of host density cycles (Voutilainen et al., 2016).

Hantaviruses are absent in some geographic locations in which potential host rodents exist, suggesting that the genetic background of the host contributes to variations in the response to infection (Rohfritsch A., 2017) (Charbonnel et al., 2014). In particular, differences in SNP allele frequencies within the TNF promoter and the Mx2 gene are likely to influence PUUV distribution and epidemiology (Charbonnel et al., 2014; Guivier et al., 2010).

1.4.2 Enzootic transmission

The dynamics of hantavirus infection are correlated with the cycles of its host population (Reil et al., 2017; Voutilainen et al., 2016). As a result, hantaviruses must have attained strategies to maintain their circulation in the often highly variable seasonal population. Hantaviruses can escape innate antiviral and proinflammatory responses and persistently infect hosts. Furthermore, they can be massively and continually shed from hosts, and the virions are highly stable *ex vivo*. Variations in the levels of virus shedding have been observed depending on the hantavirus species. They can persist for several weeks in the environment, which increases the risk of transmission to humans. Recent data using wild rodents showed that viraemic phases are longer than previously described. Infected rodents continuously shed the virus approximately one week after infection in urine, faeces and saliva, but it is shed in variable amounts up to 10 months post-infection (Voutilainen et al., 2015).

According to the ‘dilution effect’ theory, low biodiversity increases the density of potential hantavirus hosts and also changes their behaviour, e.g., through increased host-reservoir encounters (Civitello et al., 2015). The dilution effect in hantavirus-host systems has been supported through experimental and observational studies, and several studies reported lower hantavirus infection rates in hosts with a higher diversity of small mammals (Civitello et al., 2015; Khalil et al., 2016; Suzan et al., 2009; Voutilainen et al., 2012).

1.5 Epizootic of hantaviruses

1.5.1 Epidemiology

‘Spillover infection’ is a single event that occurs when a pathogen from one reservoir species moves into another novel species, forming the basis of inter-species transmission (Daniels et al., 2007). Humans acquire hantaviruses via ‘spillover infection’ by close contact with infected rodents through the inhalation of infectious aerosols of rodent excreta (Clement et al., 1996). This inhalation occurs during activities such as farming, woodcutting or the cleaning of summer cottages (Heyman et al., 2009). Other mammalian species (predatory animals), including cats, owls, dogs, and coyotes, can be infected through direct contact with rodent hosts, but they are not known to transmit the virus (Romero et al., 2003). Arthropod-like mites play a role in the maintenance and transmission of Hantaan virus and possibly other hantaviruses in nature (Yu and Tesh, 2014). Notably, human-to-human nosocomial transmission has exclusively been observed with the South American Andes virus (Martinez-Valdebenito et al., 2014; Padula et al., 1998), highlighting the potential for hantaviruses to evolve from a spillover zoonotic disease into an emerging human pathogen, causing epidemics.

In Asia, HFRS caused by HTNV and Amur/Soochong virus can reach mortality rates as high as 15%, whereas infections with SEOV result in moderate disease course with case fatality rates of 1–2% (Avsic-Zupanc et al., 2015). In Europe, NE caused by PUUV occurs throughout central and northern Europe (Vapalahti et al., 2003). The severe cases of HFRS caused by DOBV are restricted to the Balkan region (Antoniadis et al., 1996). Another genetically close strain, SAAV, can cause a mild form of HFRS in Russia, Germany and Slovakia (Klempa et al., 2003a). New World viruses, including SNV and ANDV, can result in HPS with epidemic fatality rates as high as 40% (Jonsson et al., 2010). Today, approximately 50,000 HFRS cases are reported annually. China has the largest number of cases (70–90% of all HFRS cases) worldwide (Zhang et al., 2004). Judging from the seroprevalence rates, many asymptomatic, mild or unspecific infections cases probably are neglected in clinics (Avsic-Zupanc et al., 2015). Outbreaks of sporadic cases occur every now and then (Wilken et al., 2015). Outbreaks of hantavirus infection are always linked with the population dynamics of reservoir rodents and climate change in

Asia, Europe, and the Americas (Clement et al., 2009; Luis et al., 2010; Tian et al., 2017a; Tian et al., 2015).

1.5.2 Host population dynamics

Human cases of HPS or HFRS are associated with increased contact between humans and rodent excrement, which can occur during periods of high rodent density (Milhano et al., 2017; Sane et al., 2016). The density of rodent populations is very much affected by variations in environmental factors, such as forest cover, vegetation type, food supply, and predator abundance (e.g., bank vole population dynamics), and consequently, NE epidemiology differs between biomes in Europe (Vaheri et al., 2013a). In temperate forests, masting (heavy seed crops of deciduous trees) increases bank vole densities (Tersago et al., 2009), whereas in boreal forests, population cycles of bank voles are more influenced by predation (Henttonen, 1985). Protective snow cover and temperature affect transmission rates through their effect on reproductive success and population densities. Host population dynamics are also associated with anthropogenic pressures (Armien et al., 2016).

1.5.3 Environmental changes

Environmental changes are caused by climatic, ecological and environmental changes or changes in human activities with nature or agriculture. Environmental changes give rise to the re-emergence of known hantaviruses and lead to the emergence of new viruses from a plethora of small mammal species (Klempa, 2009; Prist et al., 2016; Tian et al., 2017a; Voutilainen et al., 2016). Two main factors, temperature and rainfall, play an important role in the ecology of hantavirus (Tian et al., 2017b). Temperature can influence the reproduction and survival rates of small rodents, rodent abundance and vegetation growth (Jiang G, 2011; Prist et al., 2017). Higher temperatures have been associated with hantavirus outbreaks (Klempa, 2009; Prist et al., 2016) because of higher transmission rates or inhalation in both humans and rodents. Positive associations have also been observed between precipitation, population size of rodent hosts and prevalence of hantavirus. High precipitation increases vegetation growth, boosting rodent densities and enhancing the probability of encounters between humans and infected rodents (Xiao et al., 2016; Yates et al., 2002).

1.5.4 Disease prediction

Sophisticated models can be constructed and used to predict disease outbreak and further prevent the transmission of hantavirus to humans (Olsson et al., 2009). Remote sensing (satellite photography), together with geographic information systems characterizing areas regarding vegetation type, elevation, slope and hydrologic features, have been used to estimate the infection status of deer mice with 80% accuracy, and subsequently, also assess the risk of human infections (Boone et al., 2000). Similarly, efforts to correlate landscape characteristics and the occurrence of HFRS have been made in China (Zhang et al., 2009). Haredasht et al. have built a model-based predication of NE outbreaks in Finland and Belgium using climatological and vegetation data and bank vole population dynamics. NE outbreaks could be predicted 3 months ahead with 30–40% relative predication error (Haredasht et al., 2013).

1.6 Hantavirus and evolution

As a RNA virus, hantaviral genomes evolve with relatively low speed and strong stabilizing selection (Zhang and Holmes, 2014). The principal mechanism generating genetic diversity is genetic drift, i.e., gradual accumulation of point mutations, mostly neutral or quasi-neutral, and small deletions/insertions in the noncoding regions (Sironen et al., 2001). Reassortment of genomic RNA segments and to a lesser extent, recombination, may have also contributed to the evolution of hantaviruses (Bennett et al., 2014).

1.6.1 Genetic drift

Most RNA virus populations exist as complex mixtures of genetic and phenotypic variants, often referred to as quasispecies populations. This mixture results from a high RNA polymerase error rate, which results in misincorporation frequencies of 10^{-4} to 10^{-5} per base site on average and the apparent absence of any error correction or proofreading mechanism. This quasispecies concept has been experimentally analysed for hantaviruses *in vitro* and in animal models and experimentally and naturally infected wild-rodent hosts *in vivo* (Chung et al., 2007; Feuer et al., 1999; Lundkvist et al., 1997a; Plyusnin et al., 1995; Plyusnin et al., 1996; Sironen et al., 2008a). The theoretical advantage of maintaining a diverse quasispecies is that when the virus shifts to a

new environmental niche or selective regimen, a variant may already be present in the population that will be more fit in the new environment. However, excessive diversity can create problems if the virus is subjected to repeated bottlenecks. Since most mutations are deleterious, frequent bottlenecks can result in the rapid loss of fitness known as Muller's ratchet (Muller, 1964). Genetic diversity in RNA virus quasispecies is controlled by host-virus interaction. In addition, limitations on the genome size of RNA viruses necessitate that many viral proteins/structure carry out multiple functions, constraining their evolutionary potential (Holmes, 2003).

1.6.2 Reassortment and recombination

As hantaviruses have tri-segmented RNA genomes, they have also been subjected to reassortment events. Genetic reassortment/recombination have been detected between different hantavirus lineages/strains within the same host species. In nature, reassortment events have been found in SNV, PUUV, HTNV, SEOV, and MJNV (Kim et al., 2016a; Lee et al., 2017). Furthermore, reassortants of DOBV have been generated *in vitro* and *in vivo* (Kirsanovs et al., 2010; Klempa et al., 2003b). In addition to intra-species reassortment/recombination, reassortment/recombination can also happen between different hantavirus species. This reassortment requires double infection of an animal (or more precisely, the same cell) by two different hantavirus species. This scenario requires spillover infection of a non-reservoir animal and an accompanying infection of the same animal by the adapted host-specific virus. *In vitro* inter-species reassortment events have been reported for sigmodontine-borne BCCV/SNV and arvicoline-borne PUUV/PHV (Handke et al., 2010; Rodriguez et al., 1998). Natural inter-species reassortment between SEOV and HTNV has been detected in *Rattus norvegicu*s from China (Zou et al., 2008a). Intragenomic recombination has been reported for rodent-borne hantavirus TULV, PUUV, HTNV, ANDV *in vivo* (Sibold et al., 1999; Sironen et al., 2001; Szabo et al., 2017b) and for soricomorph-borne hantaviruses MJNV and NVAV (Kang et al., 2009c; Lee et al., 2017).

1.6.3 Evolutionary interplay between viruses and hosts

According to phylogenetic trees, five clearly distinct and well-supported groups are formed (Figure 3): (Phylogroup IIb) from shrews or moles surprisingly share a more recent ancestry with Muridae-

associated hantaviruses (Phylogroup IIa: HTNV, and alike) instead of showing a close relationship with shrew-, mole-, or bat-borne hantaviruses (Thottapalayam, Nova, Longquan, Altai-like and other viruses) from phylogroup I. The other three groups are Phylogroup III: Cricetidae-associated viruses, including two sub-groups of Arvicolinae-associated viruses (Phylogroup IIIb: PUUV, and alike), and Sigmodontinae/Neotominae-associated viruses (Phylogroup IIIa: Sin Nombre virus, SNV, and alike). Instead of forming monophyletic viral clades corresponding to the host taxa (Guo et al., 2013a), the incongruence often found in phylogenies of hantaviruses and their hosts suggests a complex evolutionary history of hantaviruses (Henttonen et al., 2008).

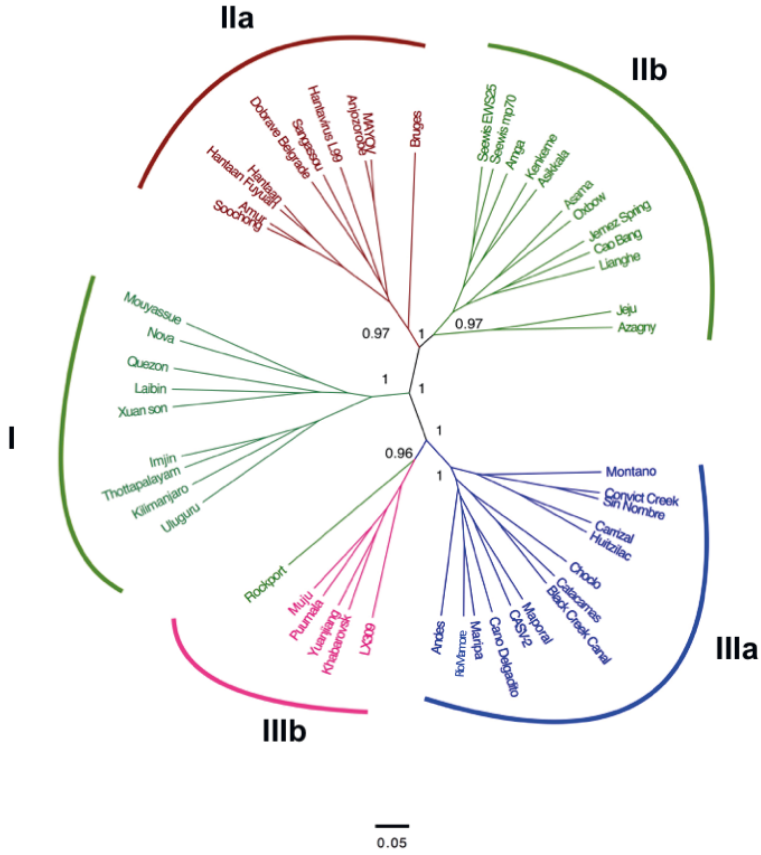

Figure 3. **Bayesian maximum cade credibility (MCC) tree based on the partial L segment sequences of representative hantavirus species.** The Bayesian posterior probability (BPP) values are given at the nodes. The scale bar shows substitutions per unit of time. Colours indicate shrew-, mole-, and bat-borne viruses (green), Murinae-borne viruses (red), Arvicolinae-borne viruses (pink), and Sigmodontinae/Neotominae-borne viruses (blue).

Red Queen hypothesis

> "Now, here, you see, it takes all the running you can do to keep in the same place," the Red Queen said to Alice in Through the Looking Glass by Lewis Carroll.

The "Red Queen" hypothesis proposed by Leigh Van Valen is that the constant extinction rates in the species population over millions of years are caused by co-evolution of species (Van Valen, 1973). The essence of the idea is that in tightly co-evolved interactions due to perturbations of one species (e.g. a predator or pathogen), another species (e.g., a prey or host) must change. Van Valen named the idea the "Red Queen hypothesis" or Law of Extinction. Species had to "run" (evolve) to stay in the same place (extant), and this scenario is a zero-sum game. Hantaviruses are highly evolved biological entities with an organismal biology that is complex and interwoven with the biology of their hosting species. This 'arms race' between host and hantavirus has been shaping hantavirus diversification in their respective hosts and/or geographic locations.

Co-evolution

Hantaviruses have co-existed with their hosts for hundreds of millions of years. This relationship represents a long-standing history of co-divergence between a host and a pathogen with a splitting time potentially dating back to the early Cretaceous period, which is the splitting time of rodents and Eulipotyphla (Plyusnin and Sironen, 2014a). This hypothesis is supported by the following evidence: geographic clustering of genetic variants (Saxenhofer et al., 2017; Souza et al., 2014; Torres-Perez et al., 2011) and high topological similarities that exist between the phylogeny of hantaviruses and hosts, especially for hantaviruses from Muridea (Hjelle, Lee, et al. 1995; Plyusnin et al. 1996; Morzunov et al. 1998; Monroe et al. 1999; Vapalahti et al. 1999; Hughes and Friedman 2000; Plyusnin and Morzunov 2001; Jackson and Charleston 2004; Nemirov et al. 2004), but not in New World hantaviruses (Rivera et al., 2015).

This hypothesis has been challenged by different studies: studies dating the emergence of insectivore-borne hantaviruses have supported different timescales from host evolution (Ramsden et al., 2009), and increasing cases of incongruent phylogenies between hosts and viruses have been found, especially after the discovery of non-rodent-borne

hantaviruses (Kang et al., 2011a; Kang et al., 2016). Instead, similarities between the phylogenies of hantaviruses and their mammalian reservoir hosts are interpreted as being due to local host-specific adaptation and preferential host switching during more recent years (Ramsden et al., 2009). However, if a virus has been co-diverging with its hosts, virus phylogeny should be similar, but not identical, to that of its hosts over the longer term, and it is more related to prior analysis or the models chosen for analysis (Sharp and Simmonds, 2011). The discrepancy could be that first, species evolutionary history and co-diverging virus phylogeny may not be accurately inferred from genomic data. For instance, in terms of the host, genetic data cannot be used as a sole indicator for tracing evolutionary history (Mackiewicz et al., 2017); in terms of the virus, full genome sequences are preferably required for evolutionary study (Szabo et al., 2017a). Second, due to the inadequacy of representative viruses or hosts, recent sporadic cross-clade transmissions or even spillover events may be superimposed upon a past history of co-evolution (Sharp and Simmonds, 2011).

In order to decipher the mechanisms of viral evolution, the evolutionary rates or fixed mutations of genomes will be estimated using nucleotide sequence data and dates of sample collection. Different conclusions were reached by various methods because they relied on different sets of assumptions; however, all the conclusions were postulated on the same basis of an approximately uniform rate of change over time. This assumption is the "molecular clock hypothesis," which means a steady accumulation of genetic changes/mutations over time (Zuckerkandl, 1962). Even recent relaxed molecular clock methods, which take into account rate variation across lineages, implicitly assume an almost clock-like evolution (Drummond et al., 2006). Molecular clock-based estimates that show a very recent time-scale with wide confidence intervals of inter-specific hantavirus evolution are likely to be erroneous or even wholly incorrect (Zhang and Holmes, 2014). Clock-like evolution is challenged by the length of the timescales. Usually, available viral sequences are very recent and many mutations have not been fixed as most viruses, such as hantaviruses, are under very strong purifying selection (Castel et al., 2014). This strong selection might lead to rejection of the clock hypothesis. As a result, calculations using recent viral gene sequences combined with common models can lead to severe underestimations of divergent time points for ancestors. For viruses, fossil records are not available, but

other sources of information can be used to estimate their substitution rates, such as calibration by epidemiological information, sequences of old isolates, or biogeographical events (Castel et al., 2017), and this information can even be combined with spatial data (Saxenhofer et al., 2017).

Cross-species transmission and host shifts

Cross-species transmission has played a central role in evolution for all virus families (Geoghegan et al., 2017) and for viral disease emergence (Parrish et al., 2008). Several cross-species transmission events have occurred during the evolution of hantaviruses: Topografov virus was isolated from *Lemmus sibiricus* (Vapalahti et al., 1999), Saaremaa virus was isolated from *Apodemus agrarius* (Nemirov et al., 1999), Dabieshan virus was isolated from *Niviventer confucianus* (Wang et al., 2000; Lin et al., 2012a), and Limestone Canyon virus was identified in *Peromyscus boylii* (Sanchez et al., 2001). Additionally, host sharing events were found in hantaviruses, which means one virus can exist in more than one species, such as SEOV in several rat species (*Rattus norvegicus*, *Rattus flavipectus*, *Rattus losea*, and *Rattus nitidus*) (Wang et al., 2000; Zhang et al., 2010a,b; Lin et al., 2012b). Examples of host sharing or spillover have been found for SWSV in the Eurasian pygmy shrew, tundra shrew (*Sorex tundrensis*), large-toothed Siberian shrew (*Sorex daphaenodon*), and Mediterranean water shrew (*Neomys anomalus*) (Resman et al., 2013; Schlegel et al., 2012a; Song et al., 2007b; Yashina et al., 2010). In addition, one species can carry more than one virus. SWSV and Altai-like viruses can be found in common shrews. NVAV and Burges virus coexist in the same common mole population (Laenen, 2016; Ling et al., 2014).

Evidence for cross-species transmission between different families of insectivores within the order *Soricomorpha* was also found among newly identified genotypes of hantaviruses in insectivores (Arai et al., 2008; Kang et al., 2009). Hantaviruses carried by *Murinae* rodents are more closely related to some Soricidae-borne viruses than to those associated with *Cricetidae* rodents (Arai et al., 2008; Kang et al., 2009; Ramsden et al., 2009). In addition, RKPV harboured by the eastern mole is more closely related to *Cricetidae*-borne viruses rather than other insectivore-borne hantaviruses (Kang et al., 2011a). These findings suggest that cross-species transmission is a major force in hantavirus evolution and speciation (Lin et al., 2012a).

Based on the phylogenetic tree, hantaviruses harboured by soricomorphs and chiropterans are far more genetically diverse than the ones by rodents, and moreover, other members in the order Bunyavirales involve insect or arthropod hosts (Marklewitz et al., 2015). The evolutionary origins of hantaviruses suggest that the primordial hantavirus may have been an insect virus, and later, it may have adapted to an early soricomorph or chiropteran ancestor. Li et al. found hantaviruses from mosquitoes. Additional in-depth studies should be carried on to search for hantavirus-like sequences in arthropods and other terrestrial invertebrates. This investigation may provide provocative insights into the evolutionary origins of hantaviruses and their relatedness to other viruses from Bunyavirales. The central hypothesis is that we are likely still scratching the surface of hantavirus biodiversity, which is produced by a complex interplay between cross-species transmission and co-divergence over long evolutionary timescales. Further studies with larger datasets could help confirm the findings and provide further insights into virus evolution (Bennett et al., 2014).

1.7 Virus isolation and diagnostic methods

1.7.1 Virus isolation

Hantaviral tropism

In a general sense, viral tropism refers to the ability of a given virus to productively infect a particular cell (cellular tropism), tissue (tissue tropism) or host species (host tropism). Hantaviruses show very similar tissue tropism in both human and rodent hosts, with consistent involvement of the vascular endothelium of the heart, kidney, lung, and lymphoid organs. The central nervous system is rarely targeted (Green et al., 1998). The tissue tropism of insectivore-borne hantavirus is similar to rodent-borne hantaviruses, but the predominance of organs involvement is different (see Section 1.4). Rodent-borne hantaviruses productively infect endothelial cells of the vasculature. The knowledge that cellular tropism of insectivore-borne hantaviruses is hampered by a lack of isolates. We found that SWSV could infect/enter endothelial cells, including human dermal microvascular endothelial cells (HDMEC) and human pulmonary microvascular endothelial cells (HPMEC) (our unpublished data). Integrins are the main receptors for rodent-borne hantaviruses *in vitro*; however, there

is little evidence to support this role *in vivo*, and it is possible that the natural hantavirus receptor is not an integrin (Gavrilovskaya et al., 1998). To date, no receptor for hantaviruses has been defined nor suggested in animal host species. Therefore, the mechanisms of viral entry in reservoir animals remain unknown (Ermonval et al., 2016).

Isolation strategies

Hantaviruses are notoriously difficult to isolate. The success of viral isolation is not related to pathogenicity. The prototype hantavirus HTNV was successfully propagated in the natural reservoir host *Apodemus agrarius*, but cell adapted isolates were obtained later (Lee et al., 1978). In addition, SNV was initially passaged in *P. maniculatus* and TULV in laboratory-colonized *M. arvalis* and thereafter propagated in Vero E6 cells. In contrast PUUV, DOBV, BCCV, TOPV and TPMV have been cultivated and isolated in Vero cells using tissue samples from naturally infected animals. DOBV, HTNV, PUUV and ANDV strains have also been isolated from infected human patients (Xiao et al., 1994). Hantavirus infection has been studied in a suckling mice model and other models, including a Syrian hamster model and Cynomolgus macaques (Brocato et al., 2014; Safronetz et al., 2014; Sironen et al., 2008b). Lethal disease was found in infant and juvenile Syrian hamsters experimentally infected with Imjin virus.

Although Thottapalayam virus, Imjin virus, and Nova virus have been isolated from the Asian house shrew, Ussuri white-toothed shrew, and European common mole, respectively, no other newfound hantaviruses have been successfully isolated in cell culture (Gu et al., 2016b). Primary and immortalized cell lines derived from reservoir hosts could provide a benefit for virus isolation, as shown in rodent-borne hantavirus. Hokkaido virus can be isolated from MRK101 cells, which are kidney cells from the grey red-backed vole *Myodes rufocanus*, the reservoir host of the virus (Sanada et al., 2012).

Several examples provide evidence that reservoir-derived cells can be beneficial over conventional cell lines for isolating reservoir-borne viruses. The henipa-related paramyxovirus Cedar virus was first isolated in primary kidney cells derived from a flying fox (*Pteropus alecto*), the species which naturally harbours this virus (Marsh et al., 2012). A new permanent cell line derived from the bank vole as a cell culture model was susceptible to several arbo- and robo-(rodent-

borne) viruses, and most interestingly, had difficulty propagating Borna disease virus (BDV) (Essbauer et al., 2011). Other new *in vitro* models, such as a three-dimensional organotypic human lung tissue model, have been used for ANDV infection (Sundstrom et al., 2016).

1.7.2 Serology

The gold standard for serotyping of hantavirus infection is the focus reduction neutralization test (FRNT) (Lundkvist et al., 1997b). However, FRNT requires special technical skill and a biosafety level-3/-4 laboratory, and thus it is not widely applied. Alternately, a system that can express glycoproteins in virus-like particles with a reporter can be used for diagnostics (Paneth Iheozor-Ejiofor et al., 2016). Also, indirect detection of hantavirus-specific antibodies is based on different serological assays, such as indirect immunofluorescence assay (IFA) and immunoglobulin (Ig)G and IgM enzyme-linked immunosorbent assay (ELISA) (Brummer-Korvenkontio et al., 1980; Lee et al., 1978). Virus preparations are required for IFA, and the florescence pattern is granular (Kallio-Kokko et al., 2001) (Lederer et al., 2013); recombinant antigens, usually N protein, are produced in different expression systems, including bacterial, yeast, insect and mammalian cells, or synthetic peptides are used for ELISA (Yoshimatsu and Arikawa, 2014a). In addition, a hantavirus point-of-care test (POC) was developed for the detection of antibodies in rodents or human (Hujakka et al., 2001; Koishi et al., 2016). A novel rapid serodiagnostic test for acute PUUV infection utilizing Förster resonance energy transfer (FRET) was recently established (Hepojoki et al., 2015).

Immunological investigations in shrews and moles are limited by the lack of commercially available species-specific secondary anti-IgG antibodies. To develop serological tests for insectivore-borne hantaviruses, a capture ELISA using a recombinant TPMV N fusion protein with an E5/G6 epitope and HRP-conjugated protein A was created. Serological surveillance has shown the presence of antibodies to TPMV in a febrile patient in Thailand (Okumura et al., 2007). Schlegel M. et al. generated monoclonal antibodies (MAbs) against TPMV and established the ELISA method (Schlegel et al., 2012b). By using the antigens from African shrew-borne hantavirus BOWV and ULUV, specific anti-shrew-borne hantaviruses antibodies can also be found in humans (Heinemann et al., 2016).

1.7.3 RT-PCR

RT-PCR attempts using S, M and L segment genome specific primers have been used not only for the detection of known hantaviruses but also for the discovery of new hantaviruses, especially with an RT-nested-PCR approach (Pan L PCR) (Klempa et al., 2006). Multiple findings of other new hantaviruses in previous years were realized by the establishment of large panels of different insectivore-borne hantavirus universal primers and touch-down PCR protocols (Kang et al., 2009a; Kang et al., 2009b; Kang et al., 2011a; Kang et al., 2009c). A probe-independent RT-qPCR (SYBR Green) assay has been generated for the detection of rodent-borne hantaviruses DOBV, HTNV, SEOV and PUUV (Lagerqvist et al., 2016). These assays have limitations in detecting viruses; when the virus genome sequences are too variable, hybridization of the probe could consequently fail due to the use of hantavirus-specific DNA probes.

Next-generation sequencing (NGS) provides an advanced tool for massive genomic sequencing of viruses and hantaviruses (Kim et al., 2016b; Song et al., 2017). By using NGS technology based on the Illumina or 454 platforms, the full genomes of Seoul, Hantaan, and Nova have been recovered (Kim et al., 2018).

1.8 Clinical features and pathogenicity

Both HFRS and HCPS are acute infections affecting the renal, cardiac, and pulmonary systems. In most cases, the infection may be asymptomatic or mistaken as ‘flu-like’(Vaheri et al., 2013c). To unify this broad spectrum of clinical symptoms, ‘hantavirus disease’ has been proposed (Clement et al., 2012). Thrombocytopenia, vascular permeability and intravascular coagulation are commonly observed in hantavirus-mediated diseases. The mechanisms of disease pathogenesis are related to the direct effects of the virus on the endothelium and by immunopathology caused by the activation of innate and adaptive immune systems in response to the virus (Jonsson et al., 2010).

Rodent-borne hantaviruses can cause diseases as clinically disparate as HFRS and HPS, which raises the possibility that newfound hantaviruses detected in shrews, moles and bats may similarly cause a wide spectrum of disease. However, the pathogenesis study on shrew-borne hantaviruses was very limited. By comparing the profiles of proin-

flammatory responses in human macrophages, Imjin virus, but not Thottapalayam virus, induces a response that is similar to the response induced by pathogenic Hantaan virus, suggesting that shrew-associated hantaviruses have the potential to cause disease in humans (Shin et al., 2012).

2 Aims of this thesis

Most recently discovered hantaviruses have been found in soricomorph and bat hosts instead of rodents, raising the question as to whether these viruses will emerge as human pathogens. Therefore, to predict human exposure risk and evaluate disease mitigation strategies, we searched for new, unknown hantaviruses from soricomorphs and bats in Finland. In this project, we gained new insights on the ecology, evolutionary origins and phylogeography of soricomorph-borne hantaviruses, and their pathogenicity. In particular, we were aiming the following:

- Sought for novel hantaviruses in soricomorphs. (Paper I)
- Reconstructed the evolution of soricomorph-borne hantaviruses in Finland. (Papers I and II)
- Mapped the serological prevalence of Seewis hantavirus infection among patients, domestic and wild animals. (Paper III and unpublished data)
- Examined the ecology of Seewis hantavirus in its host, the *Sorex araneus* population. (unpublished data)

3 Methods

3.1 Sampling of shrews (Paper I and II)

Shrews were collected from 2001 to 2015 in several field studies taking place at 45 locations/municipalities across Finland. The shrews were collected from the annual monitoring field trip by the Natural Resources Institute Finland (LUKE) and Early Warning Sample Collection with the support of EU grant FP7-261504 EDENext (http://www.edenext.eu). Traps were set on each site over two nights. Species were identified by external characteristics combined with cytochrome b (cyt-b) sequencing. The cyt-b gene of mitochondrial DNA was amplified by previously described primers (Kang et al., 2009a). Animals were dissected on the same day with immediately frozen samples, or animals were frozen on dry ice in the field and later dissected. During dissection, the animals were sexed and weighted, and the heart and lungs were placed in separate vials. Lung samples were stored at −70 °C, and the hearts were stored in PBS at −20 °C.

Permit (7/5713/2013) for capturing protected species (all shrews are protected in Finland) was granted by the Finnish Ministry of the Environment. No ethical permission is needed for snap- or live-trapping in Finland.

3.2 Serum samples (Paper III)

Five panels of serum samples were screened for specific anti-Seewis N Protein antibodies. All serum samples were kept at −20 °C until use. The details are included in the Materials and Methods sections in Paper III.

a. The archival patient samples used in this study were initially sent to Helsinki University Central Hospital Laboratory Diagnostics (HUSLAB) either because PUUV infection was suspected (for measurement of PUUV IgM and IgG antibodies (N=486)) or for routine

screening at maternity clinics for HIV antibodies and antigen and HBsAg from pregnant females (N=98, randomly selected panel).
b. A total of 152 serum samples were collected from mammologists at the Jyväskylä meeting in 1999.
c. A total of 42 lynx serum samples were collected (from Dr. Paula Kinnunen, University of Helsinki).
d. A total of 78 Finnish owl serum samples were collected (from Dr. Erkki Korpimäki, University of Turku).
e. A total of 24 street cat serum samples were collected (from Dr. Paula Kinnunen, University of Helsinki).

The human serology study was done in accordance with the research permit granted to HUSLAB (Helsinki University Central Hospital Laboratory), Department of Virology and Immunology. All patient samples were handled anonymously.

3.3 Shrew screening (unpublished)

All lung tissue samples were tested first for the presence of hantaviral RNA using semi-nested RT-PCR, which recovers a partial L segment sequence (Klempa et al., 2006). The details are included in the Materials and Methods sections of their respective original publications (Papers I and II). For SWSV viral RNA detection, specific primers SF 5'-GCTCAGAAAAAGCTAGAAAAGGC-3' and SR 5'-TCAGCTGCTGCCATTGATTG-3' were designed for SYBR Green RT-PCR by using Quanta qScript One-step SYBR Green qRT-PCR Kit, Low ROX from Thermo Fisher.

3.4 Virus isolation (unpublished)

Mammalian and insect cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) and Sf-900™ II SFM (Life Technologies), respectively. Both media were supplemented with 10% heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. The mammalian cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2, and the insect cells (High five cells, Sf9 cells, C6/36 cells) were maintained at 27 °C without supplementary CO_2.

Viral isolation attempts were made using hantaviral RNA positive lung tissues and several cell lines, including Vero E6 cells, primary kidney cells (from *Sorex araneus*), Somi-lu cells (from *Sorex minutus*) (offered from Dr. Isabella Eckerle, University of Bonn Medical Centre), BVK 18 cells (from bank vole, host of PUUV), MRK 101 cells (from grey red-backed vole, host of Hokkaido), and C6/36 cells (insect cells), according to the same methods described in (Nemirov et al., 1999; Vapalahti et al., 1999), except for C6/36 cells, which were used at room temperature. Cells were split every 2-3 weeks and mixed with uninfected cells for at least 3 months. At day 3 and day 13 post-infection, some cells were selected for RT-PCR and IFA.

3.5 Experimental analysis

The experimental analysis can be found in the method sections from the papers included in this thesis. The details are shown in the Table 3.

Table 3. **Experimental methods in studies**

DNA/RNA work		**Paper**
RNA extraction		I, II
DNA extraction		I, II
cDNA synthesis		I, II
PCR	Primer walking strategy	I, II, III
Sequencing	Sanger sequencing	I, II, III
PCR product purification	Agarose gel extraction	I, II
thymine(T) and thymine (A) cloning		I, II, III
pJET1.2/blunt clone		I, II, III
RT-PCR	SYBR Green-based	Unpublished data
Protein work		
Truncated SWSV N expression in *E. coli*	GST-tagged fusion protein	I
Protein expression in Vero E6 cells	pcDNA3.1	III
SWSV N protein expression in baculovirus system		III

Protein purification	Glutathione S-transferase (GST)-tagged truncated N protein was purified by using glutathione Sepharose 4B beads (GE health, Germany). His6-tagged SWSV N was purified by Immobilized Metal-ion Affinity Chromatography (IMAC).	I, III
Polyclonal antibody generation	GST-tagged truncated N protein (at 1 mg/ml) was sent for immunization of rabbits to produce polyclonal antibodies (BioGenes GmbH)	I
Structure prediction	3-D structure prediction was done using the Rosetta program (http://robetta.bakerlab.org/).	III
Immunoblotting		I, III
IFA		I
ELISA	Indirect ELISA, competitive ELISA	III and unpublished data
Histopathological examination		Unpublished data
Immunohistological examination		Unpublished data

3.6 Evolutionary analysis

The evolutionary analysis can be found in the method sections from the papers included in this thesis. The details are shown in the Table 4.

Table 4. **Sequences and evolutionary analysis methods used in this study**

	Method	**Software**	**Reference**
BLAST	BLASTn	NCBI	1
Sequence assembly	SeqMan	DNAstar	2
Alignment		MAFFT*	3
Substitution models	GTR+G+r HKY+G T92+G	jModelTest MEGA	4, 5

Phylogenetic analysis	Maximum-likelihood Bayesian	MEGA, BEAST, MrBayes	5, 6, 7
Reassortment		Splits tree	8
Recombination		Splits tree	8
Selection		DataMonkey	9
Network analysis	Median-Joining	Splitree	8
Phylogeography analysis		Prisma and MEGA and R	5,10

*The alignment option was using the auto strategy (FFT-NS-1, FFT-NS-2), which is like the ClustalW.

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4 Results and discussion

4.1 Hantaviruses in Finnish soricomorphs (Paper I)

To extend the knowledge of hantavirus diversity and particularly the range of their mammalian hosts in Finland, we screened samples collected during a period of 10 years for hantaviruses (Papers I and II). The samples originated from all six soricomorph species found in Finland: the common shrew (*S. araneus*), pygmy shrew (*S. minutus*), black shrew (*S. isodon*), water shrew (*N. fodiens*), masked shrew (*S. caecutiens*), least shrew (*S. minutissimus*), and common European mole (*Talpa europaea*). Four variants of novel hantaviruses were recovered: Boginia virus, Laihia strain from *N. fodiens*, Asikkala virus from *S. minutus*, and SWSV and strains related to Altai from *S. araneus* (Figure 4). Table 5 shows hantavirus RNA prevalence in Finnish soricomorphs by species. Common shrews and SWSV are the dominating species and shrew-borne hantavirus, respectively, in Finland.

Interestingly, in Paper I, we detected not only SWSV in *S. araneus* but also highly divergent Altai-like strains, most closely related to a sequence named Altai (GenBank accession No. EU424341) reported in *S. araneus* but found in Siberia. The initial assumption was spillover infections. Nevertheless, we found two hantaviruses (SWSV and Altai-like) that can circulate in one host population in both Lohja and Uurainen, where the common shrews can be considered to form one continuous population, further showing the co-existence of these two viruses. Moreover, this host sharing of two divergent hantaviruses also exists in the European mole, which can carry both BRUV (8.7%) and NVAV (53.2%) (Laenen, 2016). These observations together suggest multiple ancient host-switch events from ancestral hosts of phylogroup I hantaviruses into the soricomorph species and subsequent spread over a large range.

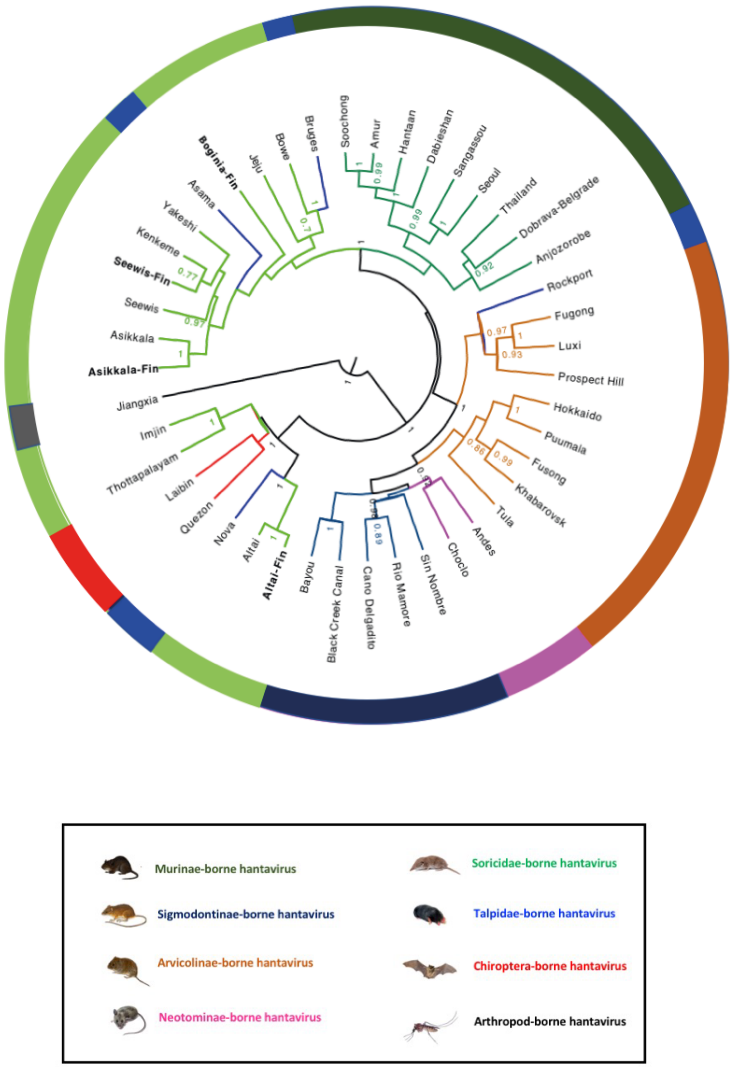


Figure 4. **Phylogeny of partial L segment sequences of selected hantaviruses.** Novel hantavirus Seewis, Boginia, Asikkala and Atlai-like viruses described in this study are shown in bold. The Bayesian Posterior probability values are given at the nodes.

Table 5. Hantavirus RNA prevalence in species

	No. of small mammals captured	Number of positive animals/total number of tested animals (%)			RNA prevalence (%)
		Female	Male	Undetermined gender	
Sorex araneus	549	38/192(19.8)	33/244(13.5)	13/113	84/594(14.1)*
Sorex minutus	33	0/7	1/15	0/11	1/33(3.0)
Sorex isodon	10	0/2	0/5	0/3	0/10(0)
Neomys fodiens	35	1/7	0/7	1/21	2/35(5.8)
Sorex caecutiens	35	0/4	0/4	0/27	0/35(0)
Sorex minutissimus	9	-	-	0/9	0/9(0)
Talpa europaea	4	-	0/1	0/3	0/4(0)
Total	675	39/212(17.0)	33/276(15.3)	14/187(7.5)	86/675(12.7)

*Of 594 common shrews, 115 were subjected to specific SWSV-specific qPCR, and 8 out of 115 (7.0%) were positive.

4.2 Genetic characterization of Finnish shrew-borne hantaviruses (Paper I and II)

We sequenced the full-length S and M segment sequences of strain EWS25 (Tammela, Southern Finland, 2012). The S segment was comprised of 1,641 nucleotides (nt). The 3'-terminal nucleotide sequence (3'-AUCAUCAUACGAGGG) was complementary to the 5'-terminal sequence (5'-UAGUAGUAGACUCCC). The S segment ORF was 1,290 nt long (corresponding to positions 47–1,336 on the S segment of strain SWSVmp70, GenBank Acc. No. EF636024), encoding a putative N protein of 429 amino acids (aa). The M segment sequence consisted of 3,533 nt, and it had a single ORF (41–3,460 nt) encoding a putative GPC protein of 1,139 aa. A putative signal peptide of 23 aa in the beginning of the ORF and the 648WAASA652 motif determining the cleavage of GPC into the Gn (630 aa) and Gc (487 aa) glycoproteins were identified (Hepojoki et al., 2012). Zinc finger domains (549–595 aa) and the 619YxxL622 motif were also identified on the glycoprotein of the EWS25 strain. Only a partial L segment sequence (1,200 nt), corresponding to positions 1,144–2,343 of the complete L segment sequence of Asikkala virus (GenBank Acc. No. KC880349) could be determined due to limited tissue material available for analysis.

The S segment of EWS25 shared 82.3% nt (98.6% aa) sequence similarity with the SWSV mp70 strain from Switzerland in 2006. Currently, there is no other complete sequence for the SWSV M segment available in the GenBank. For the partial L segment, EWS25 shared a sequence similarity of 72.3% nt (98% aa) with strain mp70. Comparison of the complete S and M segment nt and aa sequences of SWSV with those of other hantaviruses showed very low identities. The S segment identities between EWS25 and the other shrew-borne hantaviruses, Kenkeme virus (KKMV), ASIV, Yakeshi virus (YKSV), Qian Hu Shan virus (QHSV), Lianghe virus (LHEV), and Cao Bang virus (CBNV), ranged from 28.7–61.6% for the nt sequences (55.4–81.2% for aa), and the identities between EWS25 and rodent-borne hantaviruses, Puumala virus (PUUV) and Hantaan virus (HTNV), ranged from 14.8–15.1% for the nt sequences (34.1–36.6% for aa). Due to

low sequence identities, the EWS25 S segment sequence could not be reliably aligned with mole- or bat-borne hantaviruses in Phylogroup I.

The M segment of the strain EWS25 shared the highest sequence similarity with KKMV with 66.9% nt (86.4% aa). Compared to other hantaviruses, the identities were ASIV strains, 61.9–62.1% nt (84.5–84.7% aa), YKSV 61.4% nt (84.0% aa), CBNV 44.7% nt (64.0% aa), LHEV 44.0% nt (61.1% aa), and HTNV 23.5% nt (36.9% aa), and very low identities for PUUV, less than 10% nt (less than 20 % aa).

For the partial L segment, EWS25 shared a sequence similarity of 65.7% nt (93.3% aa) with KKMV, 64.7% nt (95% aa) with ASIV, 63.5% nt (86% aa) with CBNV, 55.3% nt (78.9% aa) with HTNV, and 49.3% nt (67.5% aa) with PUUV, suggesting that out of the three segments, L is the most conserved.

The strain Laihia showed 76.1% nucleotide (93.0% amino acid) identity to BOVG 2074 (reported from Poland) for the available partial 300nt L segment nucleotide sequence (Gu et al., 2013a). For the partial L segment, Asikkala showed a 79.2–81.5% nt (94.2–98.5% aa) identity to strains in the Czech Republic and Germany (Radosa et al., 2013a).

As mentioned above, SWSV and related viruses are quite divergent from Muridae-associated hantaviruses, showing up to 60% divergence at the protein level, though they shared a common ancestor. The diversity within SWSV (<10% at N protein) or with other soricomorph-borne viruses, e.g., ASIV and KKMV (18–34% at N protein, 13–16% at Gn and Gc protein), is to the similar range in rodent-borne hantaviruses (Maes et al., 2009), indicating that the shrew-borne viruses from phylogroup IIb share common features.

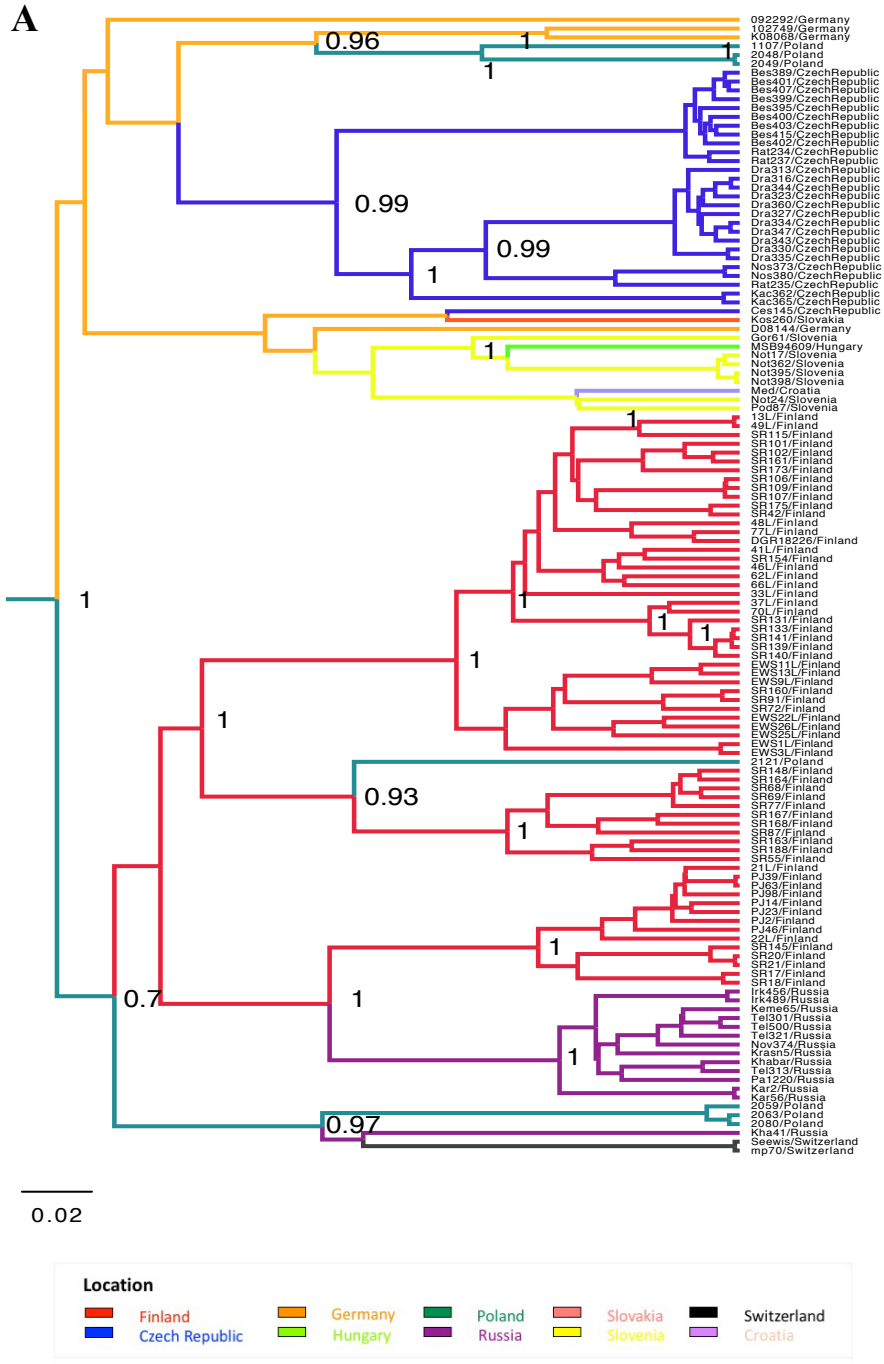
4.3 SWSV lineages and their post-glacial history (Paper II)

In order to understand post-glacial recolonization and further spread of SWSV in Finland, in paper II, we recovered the variability of Finnish SWSV and further elucidated the geographical origins, diversification and phylogeographic patterns of SWSV.

Post-glacial history of *Sorex araneus*

We had the hypothesis that Seewis is co-evolved with common shrew the same as the rodent-borne hantaviruses (Plyusnin and Sironen, 2014b) (Zhang and Holmes, 2014). This hypothesis of co-evolution between hantaviruses and their hosts is based on their co-phylogeny, as inferred from genetic markers, including chromosomal and mitochondrial phylogeny. This co-evolution has been evident, e.g., in the co-divergence study between genetic lineages of PUUV and bank voles (Nemirov et al., 2010). However, the analysis of SWSV co-evolution with its host is hampered by the fact that the chromosomal races and mitochondrial phylogeography in *S. araneus* are not congruent (Lundqvist et al., 2011). In order to test the co-evolution hypothesis, alternatively, we need to examine the biogeographical history of common shrew.

Finland is a well-studied place of phylogeography. Finland was recolonized by flora and fauna mainly from the east and southeast after the Last Glacial Maximum (LGM), when the ice retreated 17,000–10,000 years ago (Andersen and Borns, 1997) (Hughes et al., 2016) and dry land subsequently emerged. Common shrews recolonized Fennoscandia via the first land bridges from the east over Finland to northern Sweden and northern Norway. The present distribution of chromosomal races of *S. araneus* can be mostly explained by the Ancylus Lake gulf system (ca. 9,000-8,000 years ago) (Halkka et al., 1987). *S. araneus* spread first to easternmost Finland and then further north and west along the land bridges. Most of Finnish races formed during recolonization through consecutive events of Robertsonian fusion mutations (Searle, 1984). As a result, Finnish races are descendants of an old East European racial group that probably originated from east of the Urals (Halkka et al., 1994). Six of the seven Finnish chromosomal races CRs (Sa: northern Finland, Ku: central Finland, Il: easternmost Finland, Lm: south-eastern Finland, Ka: southern Finland and Le: western Finland) evolved from a common source population east of Finland (Zima et al., 1996; Wójcik et al., 2003), and they are geographically adjacent (or recently separated) populations (Hausser et al. 1994). The seventh race, Abisko (Ai), is found in northwesternmost Finland and in northernmost Sweden (Fredga 1996; Halkka et al., 1987; Halkka et al., 1994).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EWS25L/Finland
EWS1L/Finland
EWS3L/Finland
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SR69/Finland
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Kem65/Russia
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Tel321/Russia
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Krasn5/Russia
Khabar/Russia
Tel313/Russia
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Kar2/Russia
Kar56/Russia
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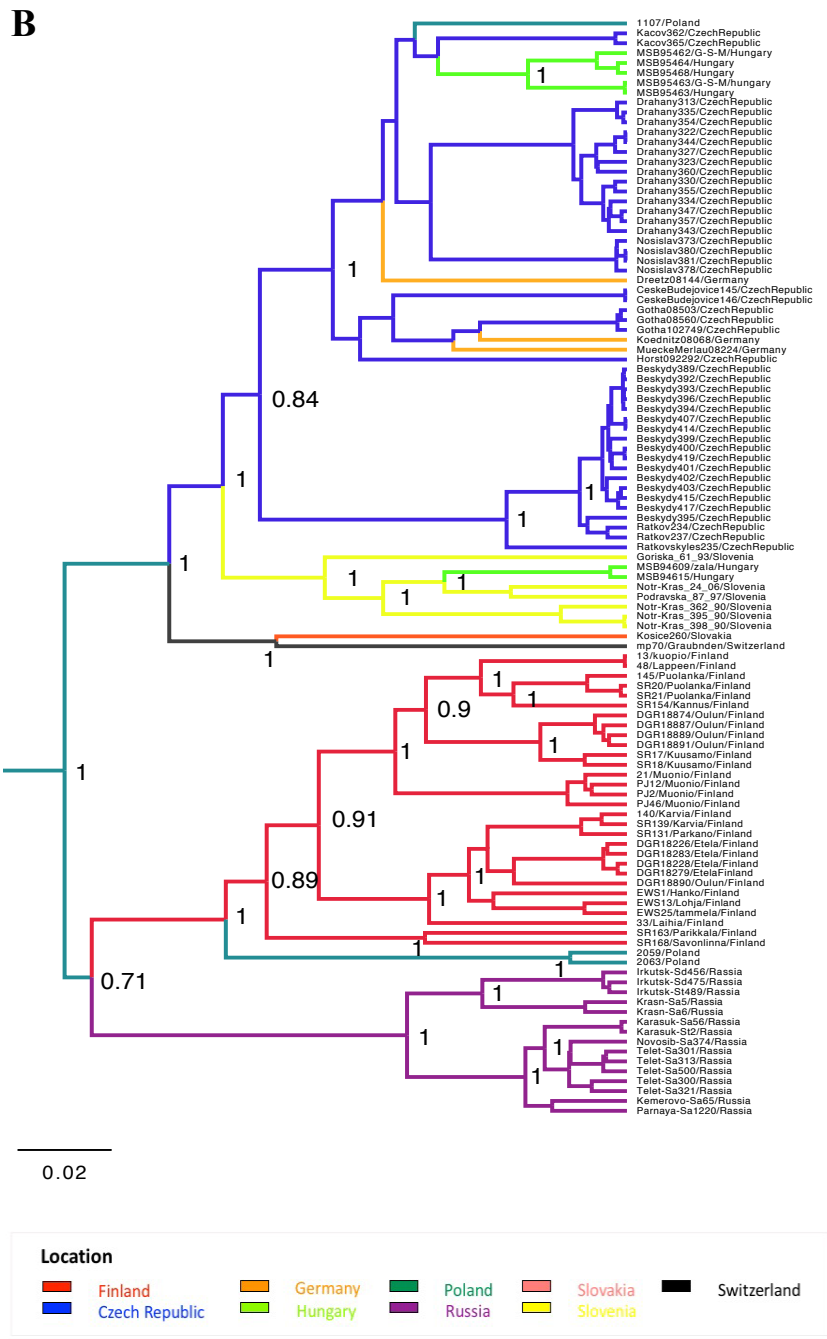

Figure 5. **Bayesian maximum cade credibility (MCC) tree based on the partial L (A) and S (B) segments of all extant SWSV.** The Bayesian Posterior probability values are given at the nodes.

Phylogeny of SWSV

The phylogeny of the S segment suggested that all Finnish SWSV S-segments (in red) formed a monophyletic group that shares a common ancestor with two Polish SWSV strains (in green) (Figure 5B). A plausible scenario would suggest that these strains share a common ancestor (potentially from Urals) that has dispersed to Europe together with its host during the last post-glacial period. An alternative hypothesis would be that Poland would have served as an ancestral refugial location for Finnish SWSV S segment lineages.

This S-phylogeny suggests that Finnish SWSV strains were introduced from eastern Finland; from this location, they underwent further dispersal to the north or south/west. This phylogeographic structure of SWSV genetic variants reflects the abovementioned post-glacial recolonization pattern of *S. araneus* (Halkka et al., 1987; Lundqvist et al., 2011), further pointing out the co-evolution of SWSV and its host *S. araneus*.

The phylogeny of the SWSV L segment showed differences from that of the S segment (Figure 5A). On the basis of the L segment, the Finnish SWSV strains formed three clusters, one of which shared (consistently with S segment phylogeny) a common ancestor with a Polish strain, whereas another shared (contradictory to S segment phylogeny) a common ancestor with Russian strains (in purple) from western and eastern Siberia (east of Urals) (Yashina et al., 2010). The Finnish strains that clustered together with the Russian strains originated from central and northern Finland, whereas the strains that clustered together with the Polish strain originated from south-eastern and eastern Finland. The third Finnish L segment cluster (with no known close relatives from other countries) contained strains from south-eastern/eastern Finland, southern Finland, and western Finland. These results suggest that the SWSV L segment may have dispersed to Finland via (at least) two routes: from east/south-east and via more norther-eastern route to central/northern Finland.

Moreover, in the southern, central, and eastern populations of Finnish *S. araneus*, SWSV reassortment events were detected, suggesting the co-circulation of different lineages possibly at the hybrid zones between chromosomal races. Chromosomal races are essentially nonoverlapping, and hybrid zones between chromosomal races are narrow, usually only a few kilometres (Andersen and Borns, 1997) (Andersen et al., 2004), but they were apparently sufficient to support reassortation.

As a result, the phylogenetic analysis on the spread of SWSV into Finland mirrors that of the host, *S. araneus*, suggesting that the Finnish SWSV, co-evolving with its host the common shrew *Sorex araneus*, colonized from the east after the last ice age (12,000–8,000 years ago) and then subsequently spread along emerging land bridges to the west or north.

4.4 Enzoonotic cycle of Seewis (Unpublished)

The common shrew is a terrestrial species living in the same ecological community with bank voles. These shrews have a very high metabolic system that requires for seeking food (insects or worms) every 2 or 3 hours. Their lifespan is short at barely 10–15 months. The breeding seasons start from May to September. Their body size can shrink during the autumn-winter with a weight loss of 12–19% to minimize the demand for food. As a result, body weight cannot be used as a sole indicator of the age. Shrews are very hard to capture in the winter. Over-wintered shrews can be captured in the spring or early summer of the next year. The predators of shrews are owls but not cats because they are foul tasting ((http://www.mammal.org.uk/))

Population dynamics and survivorship patterns of common shrews vary in different locations. In southern England, the population of common shrews showed regular cycles of abundance between seasons and years with the highest number in the summer, a decline in the late autumn and the lowest number during the winter. This cycle was not associated with ambient temperature or rainfall (Churchfield et al., 1995). However, in Fennoscandia, regular population cycles do not occur in shrews but might be interfered by rodents or predators (Henttonen et al., 1989). In our study, we found a positive correlation

between the number of common shrews we tested and the number of SWSV RNA positive shrews (Figure 6). When we tested the temporal pattern of SWSV prevalence in common shrews, the results were not significant according to the chi-square statistic. Meanwhile, by using the same statistic method, SWSV prevalence showed a spatial pattern: eastern races had a higher SWSV prevalence (35.1%) than the other races (10.7–25.0%). The chi-square statistic was 21.9747. p was 0.000203. The result was significant at $p < 0.05$. (Figure 6).

We found no significant differences in SWSV RNA prevalence between males and females or between juveniles and over-wintered shrews (Figure 8). However, from consecutive observations between 2012 to 2014, SWSV RNA prevalence is relatively low during the summer. This low prevalence is because the seasonal input of juveniles can 'dilute' SWSV infection in the old population as rodent-borne hantaviruses. Also, we could assume that maternal antibodies can protect summer-born shrews from SWSV infection, which is also similar as to PUUV infection in bank voles. Between September to November, the prevalence of SWSV increases over the time. For over-wintered shrews, we could find a relatively higher RNA prevalence than in summer-born shrews, showing a persistent infection of SWSV in common shrews (Figure 7).

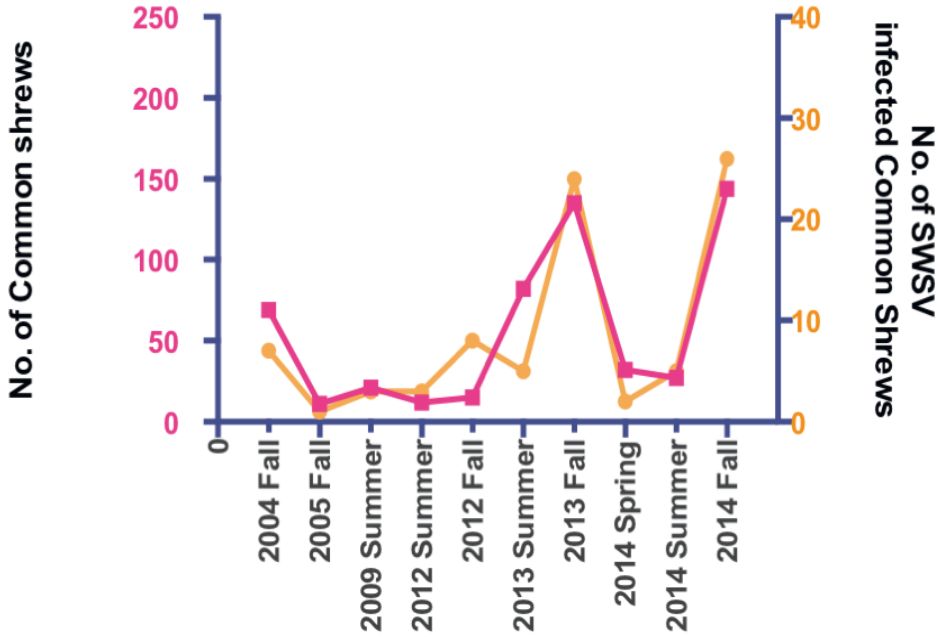

Figure 6. **The number of common shrews and the number of SWSV-infected common shrews fluctuates annually.**

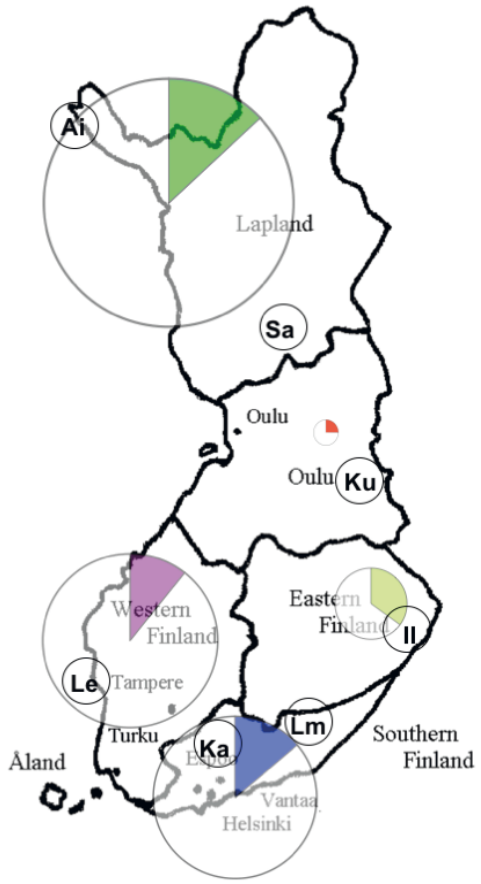

Figure 7. **SWSV RNA prevalence and distribution of Finnish SWSV in the different common shrew races.** Sa (N): northern Finland (in green) (Distribution: Lapland, N Finland), Ku (C): central Finland (in red) (Distribution: central Finland around the northern border of the central Finnish Lake District), Il: easternmost Finland (in yellow) (Easternmost tip of Finland), Lm: south-eastern Finland (in yellow) (Distribution: The southern bank of Lake Saimaa, eastern parts of the Salpausselkä Ridge, SE Finland), Il and Lm (E), Ka (S): southern Finland (in blue), Le (W): western Finland (in purple) (Distribution: Åland, the mainland of western Finland). Pie charts sizes represent the number of animals tested; the smallest chart (C)=20 individuals, and the largest chart (N)=200 individuals.

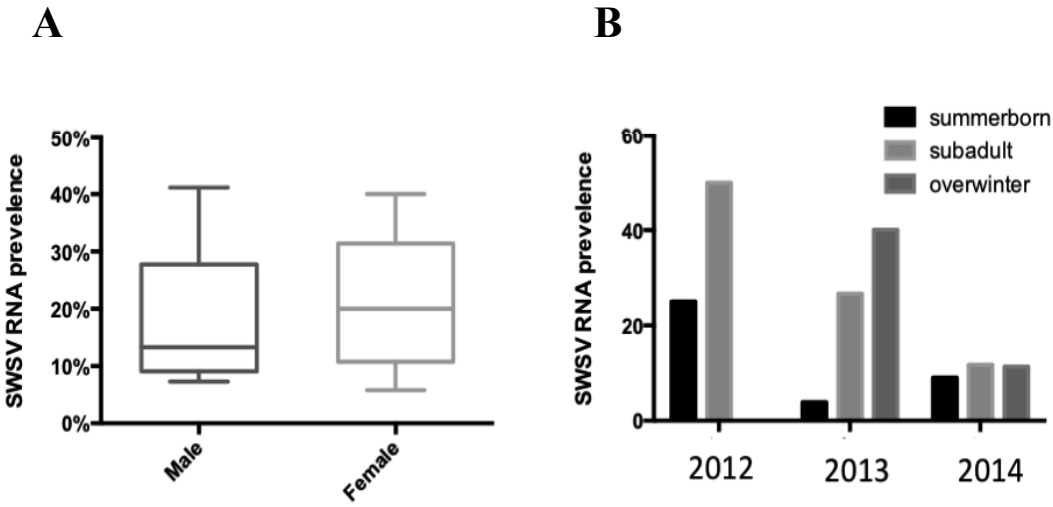

Figure 8. **SWSV RNA prevalence in male and female common shrews** (A) or in different ages of common shrews (B).

4.5 Antigenic properties of Seewis virus (Paper III)

Generation of polyclonal antibodies and antigen detection in shrews

In Paper I, we generated a polyclonal antibody to detect the SWSV N antigen. As the N-terminal 120 amino acids have been shown to be highly antigenic for rodent-borne hantaviruses (de Carvalho Nicacio et al., 2002), a truncated SWSV N protein (aa 1-120) produced as a GST-fusion protein (of around 37 kDa) in *E. coli* was used for the immunization of two rabbits.

In Paper III, a histidine-tagged recombinant SWSV N protein was produced in H5 cells. The purified protein was confirmed by immunoblotting to react with both SWSV antiserum and heart extracts of SWSV-positive shrew samples. We further characterized the recombinant SWSV N proteins in immunoblotting and IFA using a panel of hantavirus N-specific monoclonal antibodies (MAbs) 4C3, 2E12, 3C11, 3 F10, 2D9, 1F1, 2H6, 2E7, ECO2, E5G6, and F23A1, but none of the MAbs reacted with SWSV N. However, the polyclonal rabbit antiserum against the first 120 aa of SWSV N was found to react with PUUV N in immunoblotting, further demonstrating the antigenic similarity of this region. Moreover, the predicted 3-D structures of the SWSV and PUUV N protein N-terminus are homologous, both containing three helices that assemble as an antiparallel coiled-coil structure. By alignment, the first 120 aa of PUUV, TULV, HTNV, TMPV, and SWSV N proteins indeed share common linear epitopes, further suggesting that epitope mimicry would explain the observed cross-reactivity (Paper III).

Seewis virus isolation

Different cell models, including Vero E6 cell (deficiency in producing interferon), primary kidney cells (from *Sorex araneus,* the host of SWSV), Somi-lu cells (from *Sorex minutus*, the host of ASIV), BVK 18 cells (from bank vole, host of PUUV), MRK 101 cells (from grey red-backed vole, host of Hokkaido), and C6/36 cells (insect cells), have been used for SWSV isolation attempts, but unfortunately, we were unable to detect SWSV RNA or SWSV N protein in the cells or supernatant after the first passage (10 dpi) until three blind passages (over 30 days).

Also, to investigate that whether TULV, PUUV, and HTNV can enter these shrew cells, we performed the infection experiment *in vitro.* After three days post-infection, N protein of TULV, PUUV, and HTNV can be detected in Vero E6 cells by IFA; none of the rodent-borne hantaviruses can infect Somi-lu cells. This result further indicated that viral entry of shrew-borne viruses might be different from that of rodent-borne viruses. PUUV (Sotkamo strain) can infect MRK 101 cells but not BVK18 cells. The latter cell line was reported to be infected by the PUUV Vranica strain. Interestingly, we could see one or two insect cells infected by TULV or PUUV, but they had very low florescence staining.

Seewis virus in the target organs or tissues

We collected lungs, kidneys, livers, and spleens from three SWSV RNA-positive shrews (No. 13, 48, and 49) (Paper I), but were only able to detect SWSV RNA in lung tissues. This observation is distinct from rodent-borne hantaviruses, suggesting that tissue tropism might be different in shrew-borne hantaviruses. As a result, we used lung tissues for immunohistochemical staining of SWSV antigens. (Figure 9)

Lung and kidney tissues from a SWSV-positive shrew (EWS22) were collected for histology and immunohistochemistry to detect pathology caused by the infection (Figure 9). Lung sections showed some differences in SWSV-positive compared to uninfected controls. Control lungs showed clear airways (bronchiole) and air spaces, normal vessels (pulmonary artery) and normal thickness of the interstitium (B, 100×). SWSV-infected lungs showed alveolar haemorrhage, oedema, and mild interstitial pneumonia (D, 100×). Immunohistochemistry performed on SWSV-infected lung sections showed widespread immunoreactivity in the alveolar septa and endothelial cells of larger vessels (C). This observation is intriguing as it contradicts the fact that no clear pathological changes were observed between the infected or non-infected rodents. However, this result came from only one individual shrew sample.

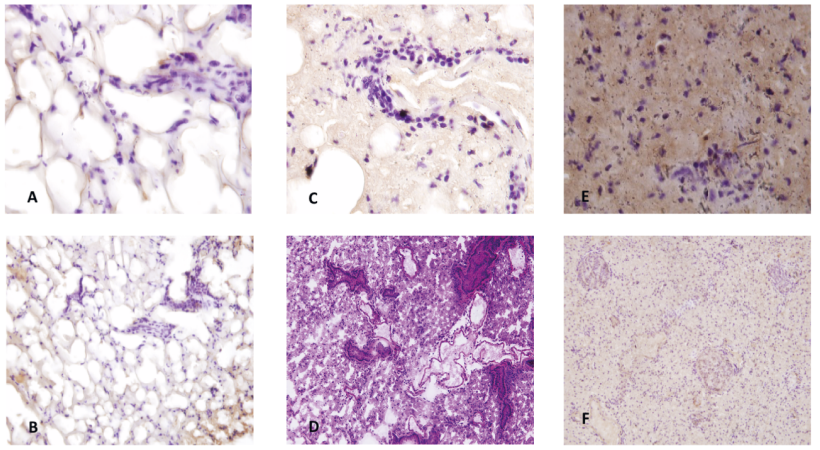

Figure 9. **Effects of hantavirus infection on shrew lung.** An alkaline phosphatase immunohistochemical assay using anti-SWSV nucleocapsid antibody was performed on lung samples from SWSV-free shrew (EWS5) (A, B) or lung and kidney samples from SWSV-infected shrew (EWS22) (C, D, E, F).

4.6 Potential pathogenicity of Seewis (Paper III)

N protein is the immunodominant structural protein of hantaviruses. The N-terminal region of hantavirus N harbours common epitopes responsible for the cross-reactivity between different hantaviruses (Yoshimatsu and Arikawa, 2014b). To investigate the potential pathogenicity of shrew-borne hantaviruses, SWSV antigens were produced for mapping seroprevalence in humans.

These serological tests (SWSV ELISA, IFA, and IB) can be used in the diagnosis of previously unrecognized hantaviral diseases. We initially screened 486 (450 PUUV-seronegative and 36 PUUV-seropositive) samples sent to Helsinki University Hospital Laboratory for PUUV serodiagnosis during 2002 and 2007 in SWSV N IgG ELISA. In total, 4.2% (19/450) of PUUV-seronegative samples were reactive in SWSV N IgG ELISA, and none of the tested samples (43 PUUV-seronegative, weakly reactive in SWSV IgG ELISA, and 15 random) were reactive in SWSV N IgM ELISA. None of the IgG re-

actions could be confirmed by IFA or IB. Furthermore, among 36 PUUV-seropositive samples, three were reactive in SWSV N IgG and ten in SWSV N IgM ELISA. One PUUV-seropositive sample reacted with SWSV N protein in IFA and four in IB. Finally, we applied competitive ELISA to confirm that the observed reactivity is cross-reaction rather than a true SWSV response. In conclusion, no evidence of SWSV infection was found among the 486 human serum samples studied; however, we could demonstrate that PUUV antiserum cross-reacted with shrew-borne hantavirus N protein.

Moreover, we screened a serum panel of occupational risk. A total of 152 serum samples from mammologists at the Jyväskylä meeting in 1999 were screened for specific anti-SWSV N IgG; again, there was no obvious positive reaction. The predators of shrews, such as street cats, lynx, and owls, were also screened for specific anti-SWSV antibodies, but the samples were negative. However, we found that a single serum sample from one owl was positive for PUUV IFA.

Taken together, we obtained no direct evidence that SWSV could infect wild animals (cats, lynxes and owls) or humans. However, we can not exclude the possibility that SWSV could spill-over and infect humans, and even cause diseases. We should always remember the lessons that we had learned from emerging HPS. That was, in 1993, a rapidly progressive cardiopulmonary disease with a mortality rate up to 40% for the first time raised the vigilance of doctors, emergency physicians and health-care workers in the southwest United States. The aetiological agent is a neotomine-borne hantavirus identified a decade earlier; however, it was beyond the realization of clinicians, epidemiologists and medical virologists.

5 Concluding remarks and future perspectives

Hantaviruses are zoonotic viruses. In Finland, PUUV was the only hantavirus detected for years. In 2009, however, SWSV was reported from Finnish archival common shrew (*Sorex araneus*) samples collected in 1982. Subsequently, Austria, Czech Republic, Switzerland, Germany, Hungary, Poland, Russia and Slovenia also reported that Seewis RNA existed in common shrews. So far, we found that four soricomorph-borne hantaviruses circulated in Finland. The clear conclusion from this study combined with other surveys on hantavirus biodiversity is that soricomorphs are important natural reservoirs for hantaviruses.

Hantaviruses are ancient viruses and co-evolved with their host. This well-established hypothesis of co-divergence of these viruses with their hosts was questioned when highly divergent novel hantaviruses were recovered globally. We found that the dispersal of SWSV coincided with the migration and population expansion of its host, evidencing that the 'co-evolution theory' also applies to shrew-borne hantaviruses. Furthermore, it is likely that the primordial host of hantaviruses was a shrew, mole, or bat or that a (pre)hantavirus was transmitted from an insect to a mammalian host (Plyusnin & Sironen., 2014).

Hantaviruses are emerging viruses. In Finland, PUUV co-circulates with SWSV, whereas PUUV causes 1,000-3,000 NE cases annually, and the pathogenicity or virulence of SWSV to man is unknown. In this study, we could not demonstrate SWSV antibodies in the studied sample panels. This result suggests that it is quite unlikely or rare that SWSV would infect or induce a disease in humans. However, the possibility of human SWSV infection cannot be completely ruled out. Moreover, serology has been the main approach to finding new viruses with disease association in the pre-gene-technology era. The initial identification of PUUV in Finland was done using a lung tissue section of bank vole that was used as antigens for the sera of nephropa-

thia epidemica patients. The serology investigation developed in this study can be used to discover additional novel related hantaviruses in future.

In conclusion, we are still capturing a small fraction of hantavirus diversity. In the age of metagenomics, many more genetically distinct hantaviruses, not only from the related host species but also from arthropods, await discovery. Besides, not only hantaviruses but the other novel viruses, especially human disease-related ones, would be discovered in shrews, or other soricormophs by using viral metagenomics analysis. More importantly, these novel viruses need to be isolated to gain new insights on the evolutionary origins and phylogeography of hantaviruses and possibly the molecular determinants of pathogenicity.

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