Recombinant hantavirus proteins: Antigenic properties and diagnostic applications

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List of original publications

This thesis is based on the following original articles referred to in the text by their Roman numerals.

I. Kallio-Kokko H, Vapalahti O, Hedman K, Brummer-Korvenkontio M, Vaheri A (1993) Puumala virus antibody and immunoglobulin G avidity assay based on a recombinant nucleocapsid antigen. Journal of Clinical Microbiology 31:677-680.

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

III. Kallio-Kokko H, Vapalahti O, Lundkvist Å, Vaheri A (1998) Evaluation of Puumala virus IgG and IgM enzyme immunoassays based on recombinant baculovirus-expressed nucleocapsid protein for early nephropathia epidemica diagnosis. Clinical and Diagnostic Virology 10:83-90.

IV. Kallio-Kokko H, Lundkvist Å, Plyusnin A, Avsic-Zupanc T, Vaheri A, Vapalahti O (2000) Antigenic properties and diagnostic potential of recombinant Dobrava virus nucleocapsid protein. Journal of Medical Virology 61:266-274.

V. Kallio-Kokko H, Leveelahti R, Brummer-Korvenkontio M, Lundkvist Å, Vaheri A, Vapalahti O (2000) Expression of Puumala virus glycoproteins and nucleocapsid protein in mammalian cells, and use in characterization of human immune responses in nephropathia epidemica. (submitted).

Abbreviations

Aphos	**alkaline phosphatase**
ANDV	**Andes virus**
bac	**baculovirus**
β-gal	**b -galactosidase**
CTL	**cytotoxic T lymphocyte**
DAB	**1-1-diaminobenzidine**
DOBV	**Dobrava virus**
EIA	**enzyme immunoassay**
FITC	**fluorescein isothiocyanate**
FRNT	**focus reduction neutralization test**
HCPS	**hantavirus cardiopulmonary syndrome**
HFRS	**hemorrhagic fever with renal syndrome**
HPS	**hantavirus pulmonary syndrome**
HTNV	**Hantaan virus**
ID	**infectious dose**
IFA	**immunofluorescence assay**
IgG	**immunoglobulin G**
IgM	**immunoglobulin M**
kb	**kilobase**
kDa	**kilodalton**
KHF	**Korean hemorrhagic fever**
MAb	**monoclonal antibody**
N	**nucleocapsid protein**
DN	**truncated nucleocapsid protein**
NE	**nephropathia epidemica**
OPD	**o-phenylenediamine dihydrochloride**
PCR	**polymerase chain reaction**
Perox	**peroxidase**
PHV	**Prospect Hill virus**
pNPP	**p-nitrophenyl phosphate**
PRNT	**plaque reduction neutralization test**
PUUV	**Puumala virus**
rN	**recombinant nucleocapsid protein**
RT-PCR	**reverse transcriptase-PCR**
SDS-PAGE	**sodium dodecyl sulfate - polyacrylamide gel electrophoresis**
SEOV	**Seoul virus**
SNV	**Sin Nombre virus**
TMB	**tetramethylbenzidine**
TULV	**Tula virus**

Summary

Hantaviruses are rodent-borne viruses with a tripartite RNA genome that are transmitted to humans through excreta of infected rodents; each hantavirus is carried by a specific rodent host. Hantaviruses cause two diseases, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS), which vary in severity depending on the causative agent. Hantaviral infections occur world-wide, but are especially common in China, Korea, Russia, and Northern Europe. In Finland alone on average one thousand nephropathia epidemica (NE) (a mild form of HFRS) cases are diagnosed annually.

One aim of this study was to develop new enzyme immunoassays (EIA) based on recombinant hantavirus antigens for hantavirus diagnosis. In Europe, two hantaviruses, Puumala virus (PUUV) and Dobrava virus (DOBV), are known to occur. Recombinant PUUV and DOBV nucleocapsid proteins were expressed in bacterial or insect cells, and based on these antigens, EIAs were developed to measure IgG and IgM antibody responses in humans. These tests were found to be highly specific and sensitive in diagnostic use. The best format for early diagnosis of acute infection was a μ-capture EIA based on baculovirus-expressed full-length nucleocapsid protein (PUUV-N and/or DOBV-N). Demonstration of IgM-class antibodies was diagnostic, but in a serum sample taken before the 6th day after onset of symptoms the IgM test sometimes remains negative and a second sample is needed. Specific assays based on antigens from viruses circulating in each geographical region improve the sensitivity of the tests.

In order to further investigate the immune responses in hantavirus infections, PUUV glycoproteins were expressed in mammalian cells using an alphavirus-derived vector. For optimal results, co-expression of the recombinant glycoproteins G1 and G2 was found to be essential. IgG antibodies against glycoproteins appeared only in the late convalescent phase in NE-patient sera, while IgG antibodies against N were seen already in the acute phase in high titers. The presence of IgG antibodies to glycoproteins leads to the diffuse type of fluorescence in native PUUV IgG-immunofluorescence assay typical of old-immunity sera.

In order to map the B-cell epitopes on N by use of monoclonal antibodies, truncated PUUV nucleocapsid proteins were used as antigens. In experimental animals, the aminoterminal part of PUUV-N was shown to be highly immunogenic, and in protection experiments, immunizations with total recombinant PUUV-N or its aminoterminal aa 1-118 (expressed in insect and bacterial cells, respectively) were able to induce protection against PUUV infection in bank voles. The recombinant proteins, DNA vector constructs, and animal model introduced here provide a valuable tool for future vaccine research.

1. Introduction

1.1. Historical background

The first descriptions of a hemorrhagic fever associated with renal syndrome were recorded in Chinese medical literature about A.D. 960 [80], but it was only in 1951 during the Korean War that the disease, named Korean hemorrhagic fever (KHF), was first encountered by western medicine [86]. The causative agent, Hantaan virus (HTNV) was isolated in 1978 from the rodent *Apodemus agrarius* and from KHF patients [82], and later propagated in a human cell line [49].

Nephropathia epidemica (NE) was first described in Sweden in 1934 [108, 169], but the causative agent, Puumala virus (PUUV), was not isolated until 1977 from a bank vole (*Clethrionomys glareolus*) captured in Puumala, Finland [23]. By that time, many NE cases had been detected and epidemiological and clinical features described [77]. During the early 1980s, virus-infected lung sections of *Clethrionomys glareolus* [23] were used as antigen for serologic diagnosis. In 1983 PUUV was adapted to cultured Vero E6 cells [115, 133], which have been the main source of antigen for immunofluorescence (IFA) and enzyme immunoassay (EIA) [23, 115].

KHF and NE are classified as two distinct forms of hemorrhagic fever with renal syndrome (HFRS), varying in the severity of symptoms. The causative agents of HFRS are called hantaviruses according to the prototype member of the genus, HTNV [133].

In the Balkans, the first HFRS cases were reported in 1952, and after that, several clinical cases have been reported throughout Eastern Europe. In 1995, Dobrava virus (DOBV), originally isolated from a yellow-necked mouse (*Apodemus flavicollis*) in Slovenia [17], was characterized genetically and antigenically [16]. DOBV is associated with severe HFRS, similar to that caused by HTNV [8, 15, 94].

In the early 1980s, Prospect Hill virus (PHV), considered apathogenic to humans, was found in the United States in *Microtinae* rodents [84, 85]. However, it was not until 1993 that hantaviruses (e.g. Sin Nombre virus (SNV)) carried by New World rodents (*Sigmodontinae*) were recognized as the causative agents of a highly lethal human disease, hantavirus pulmonary syndrome (HPS) [113], recently also named hantavirus cardiopulmonary syndrome (HCPS).

1.2. Structure and coding of hantaviruses

Hantaviruses, members of the family Bunyaviridae [133], are negative-stranded RNA viruses with a tripartite genome; the S (small) segment encodes a nucleocapsid protein (N) [134], the M (medium) segment two glycoproteins G1 and G2 [135], and the L (large) segment an RNA polymerase [147] (Table 1). Some hantaviruses, including PUUV and SNV but not HTNV and DOBV, have an additional open reading frame overlapping with that of N and coding for a putative non-structural NS_s protein.

Table 1. *Hantavirus genome and coding*

Genome segment	Size in kb	Protein	Size in kDa
S	1.7-2.0	N	~50
		NSs*	10
M	3.6-3.7	G1 and G2	~68 and ~54
L	6.5	RNA polymerase	~ 200

* Putative for PUUV and SNV; not HTNV or DOBV

The RNA molecules appear circular due to the complementary 5' and 3' ends of the segments which anneal to form panhandle-like structures. The RNA segments and nucleocapsid proteins form ribonucleoproteins which include the RNA polymerase molecule, and are surrounded by a host cell-derived lipid bilayer in which the glycoproteins G1 and G2 are embedded, forming heterodimers (Fig. 1). The two glycoproteins G1 and G2 are cotranslationally cleaved from a single precursor polypeptide. The virus particles of prototype virus HTNV mature by budding
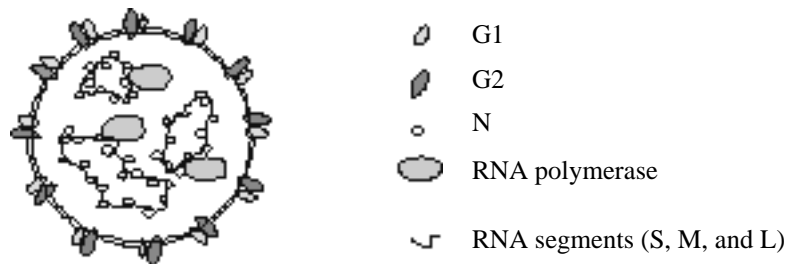

G1

G2

N

RNA polymerase

RNA segments (S, M, and L)

Figure 1. *Schematic structure of hantaviruses.*

from the Golgi complex [122]. Hantavirus particles are spherical or oval in shape, with a mean diameter of 122 nm (range 78 to 210 nm) [148].

During viral entry, the G1 and G2 glycoproteins attach to host cell proteins. Pathogenic hantaviruses can attach to β_3-integrins, which are possible receptors for these viruses, whereas apathogenic Prospect Hill virus uses β_1-integrins [51, 52].

1.3. Transmission

Hantaviruses are carried by specific rodent hosts, in which the viruses cause asymptomatic infections that can persist for several months. The virus can be detected in different organs of the animals, although the highest viral loads have been found in the lungs [50, 81-83, 85, 112, 164]. During the viremic period, the virus is transmitted to humans probably via aerosolized excreta. Among rodents, the virus is transmitted horizontally likely in the same way [18, 68, 69, 81, 164]. Maternal antibodies transmitted either *in utero* or through breast-feeding protect the animals against infection during the first weeks of life [36, 37]. Transmission from human to human does not usually occur, although for some HPS outbreaks caused by Andes virus (ANDV) in Argentina this route has been reported [117].

Animal trappers, forestry workers, farmers, and mammalogists have an increased occupational risk of contracting PUUV infection [4, 140, 155, 156, 172].

1.4. Epidemiology

In Europe, only two hantaviruses pathogenic to humans are known to circulate, namely PUUV and DOBV (Table 2a). The bank vole (*Clethrionomys glareolus*) is the carrier of PUUV [22, 23], which is found in most of Europe, with the highest incidence of human PUUV infections reported in Finland, Sweden, and Russia [1-3, 13, 24, 33, 53, 57, 59, 79, 100, 101, 116, 120, 140, 150, 172] (Table 3a). The yellow-necked mouse (*Apodemus flavicollis*) and the striped field mouse (*A. agrarius*) have been shown to carry two genetically distinct lineages of DOBV [14, 17, 111, 127]. Human DOBV infections have been reported in Albania, Greece, Bosnia-Herzegovina, Slovenia, Germany, Estonia, and Russia [8, 15, 91, 94, 100, 104]. The two genetic lineages of DOBV appear to be associated with different pathogenicity of the virus; DOBV in

*Table 2a. Human-pathogenic hantaviruses**

Virus	Abbr.	Rodent host		Location	Ref.	Disease	Mortality
Puumala	PUU	Bank vole	*Clethrionomys glareolus*	Europe	[22, 23]	NE	<0.1%
Dobrava	DOB	Yellow necked mouse	*Apodemus flavicollis*	Europe	[16, 17]	HFRS	~7%
		Striped field mouse	*A. agrarius*				low**
Hantaan	HTN	Field mouse	*A. agrarius*	Asia	[49, 82]	HFRS	2-7%
Seoul	SEO	Rats	*Rattus norvegicus*	Asia, world-wide	[83]	HFRS	<2%
			R. rattus				
Sin Nombre	SN	Deer mouse	*Peromyscus maniculatus*	North and South America	[30, 44, 114]	HPS	~40%
Andes	AND		*Oligoryzomys longicaudatus*	Argentina	[88, 89]	HPS	~40%
Black Creek Canal	BCC	Cotton rat	*Sigmodon hispidus*	North America (USA)	[129, 130]	HPS	~40%
New York	NY	White-footed mouse	*P. leucopus*	North America (USA)	[61, 145]	HPS	~40%
Bayou	BAY	Rice rat	*Oryzomys palustris*	North America (USA)	[107]	HPS	~40%

*viruses isolated in cell culture; **no lethal cases reported

Table 2b. Hantaviruses not known to be pathogenic to humans

Virus	Abbr.	Rodent host		Location	Ref.
Thailand	THAI	Bandicoot	*Bandicola indica*	Asia (Thailand)	[45]
Topografov	TOP	Siberian lemming	*Lemmus sibiricus*	Siberia	[127, 159]
Prospect Hill	PH	Meadow vole	*Microtus pensylvanicus*	North America (USA)	[84, 85]
Tula	TUL	European common vole	*Microtus arvalis*	Europe (Russia, Slovakia)	[126, 161]
			M. rossiaemeridionalis		
Isla Vista	ILV	California vole	*M. californicus*	North America (USA)	[146]
Khabarovsk	KBR	Reed vole	*M. fortis*	Asia (Far East Russia)	[64]
Thottapalayam	TPM	Shrew (insectivore)	*Suncus murinus*	Asia (India)	[28]
Rio Mamore	RM	Pygmy rice rat	*Oligoryzomys microtis*	South America	[19]

the Balkans (carried by *A. flavicollis*) causes more severe symptoms (with mortality rates of 4-12%) [15] than DOBV in Estonia and Russia (carried by *A. agrarius*), where no fatal cases have been observed [91, 100].

Other human-pathogenic hantaviruses include Hantaan virus (HTNV) carried by the field mouse (*Apodemus agrarius*), circulating in Asia, and Seoul virus (SEOV) carried by rats (*Rattus norvegicus* and *R. rattus*) [49, 82, 83] (Table 2a). SEOV has been found in rats throughout the world, while human illness has been reported primarily in Asia. In the Americas, Sin Nombre

Table 3a. *Hantavirus seroprevalence and the number of cases in Europe (PUUV/DOBV)*

Country	Seroprevalence (PUUV/DOBV)	Reference	Cases per year
Austria	1.2	[1]	<10
Belgium	?	[59]	up to 200
Bosnia	5	[13]	up to several hundred
Estonia	3 (2/1)	[101]	?
Finland	5	[24]	˜1000
France	?	[79]	up to 200
Germany	1.7	[173]	up to 200
Greece	4	[121]	10-20
Netherlands	0.9	[57]	?
Norway	?	Folkhelsa, Norway**	50-100 (1998:200)
Slovakia	0.84 (0.42/0.42)	[141]	?
Russia	1.5-4.3	[151]	2-18/100000
Sweden	5-9*	[2, 3]	50-200 (1998:500)

* Northern Sweden; ** Annual Report 1998, National Institute of Public Health, Norway, page 13

Table 3b. *Examples of reported HPS cases in the Americas*

Country	HPS cases (cumulative total)	Mortality
Argentina	>270*	~40 %
Brasil	65**	60 %
Canada	34***	38 %
Chile	123****	49 %
USA	245*****	44 %

* up to 5/2000 (Promed Mail); **up to 10/2000 (Promed Mail)***up to 6/2000 (Health Canada Laboratory Centre for Disease Control); **** up to 8/2000 (Departomento de Epidemiologica, Chile); ***** up to 6/2000 (CDC, USA)

virus (SNV) carried by deer mouse (*Peromyscus maniculatus*), and related viruses carried by other *Sigmodontinae* rodents circulate [30, 44, 61, 88, 89, 106, 113, 128, 129, 144] (Table 2a). The main pathogen causing HPS in North America is SNV, and in South America, ANDV.

Several other hantaviruses have been isolated and characterized throughout the world, but none have been shown to be pathogenic to humans [19, 28, 45, 64, 84, 85, 125, 126, 145, 158, 160] (Table 2b).

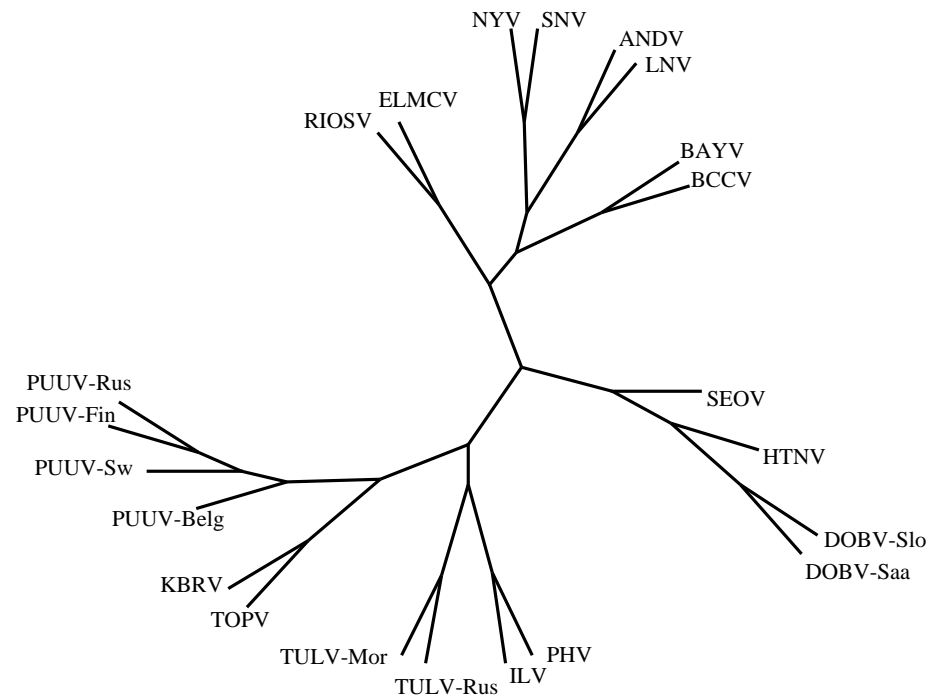The phylogeny of hantaviruses based on aminoacid sequence of the nucleocapsid protein is presented in Fig. 2 [48].

Figure 2. *Phylogenic tree of hantaviruses based on the aa sequence of N (with Neighbour Joining Method using the PHYLIP program package [49]). Rus = strain from Russia; Fin = strain from Finland; Sw = strain from Sweden; Belg = strain from Belgium;Saa = strain from Apodemus agrarius in Saaremaa, Estonia; Slo = strain from Apodemus flavicollis in Slovenia; RIOSV = Rio Segundo virus; ELMV = El Moro Canyon virus; LNV = Laguna Negra virus. Figure was provided by Alexander Plyusnin.*

1.4.1. NE epidemiology in Finland

In Finland, the incidence of laboratory-confirmed NE follows the density of rodents during different years and varies depending on the geographical location. The incidence peaks occur every 3-4 years. Clinical NE cases are detected throughout the year, but the highest numbers are diagnosed in November and December in rural populations, and in August in urban populations [24] (Fig. 3). Eastern Finland has in general the highest rates (Fig. 4).

Males are infected at a mean age of 40 years and females at 44 years (Fig. 5), and the male:female incidence ratio is about 2:1. The number of serological diagnoses (19/100 000) and the antibody prevalence (5%) indicate that at least 70% of PUUV infections remain undiagnosed [24].
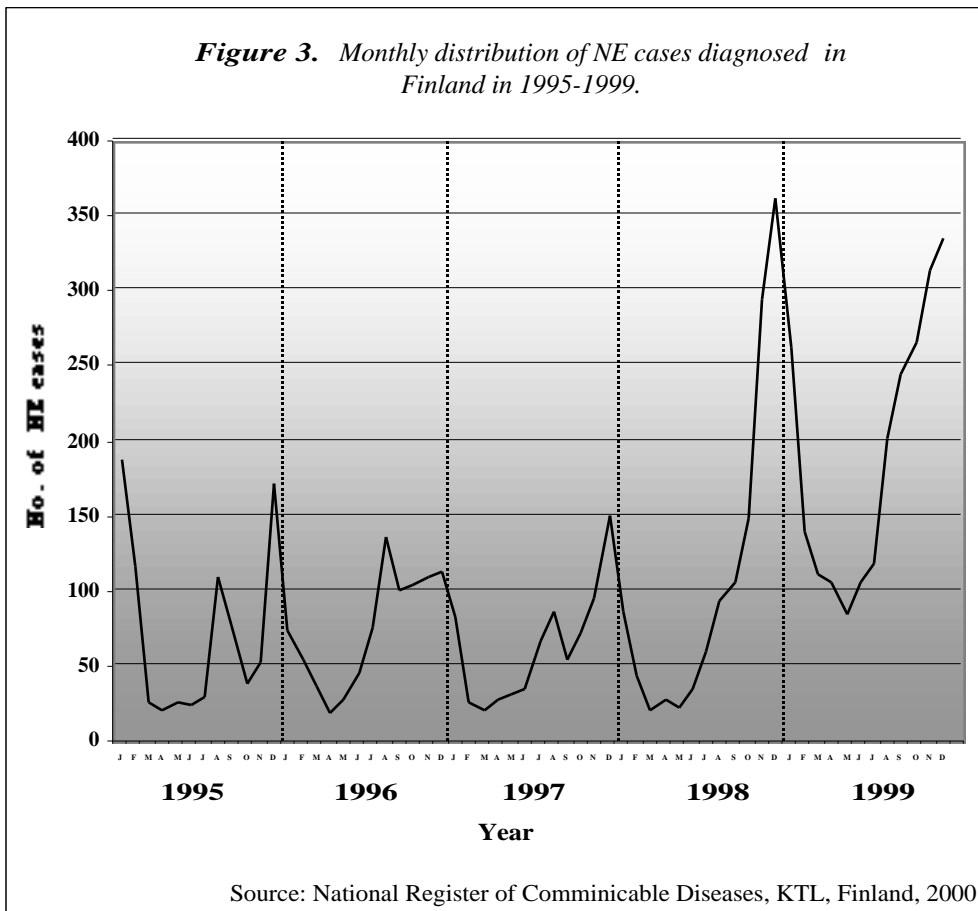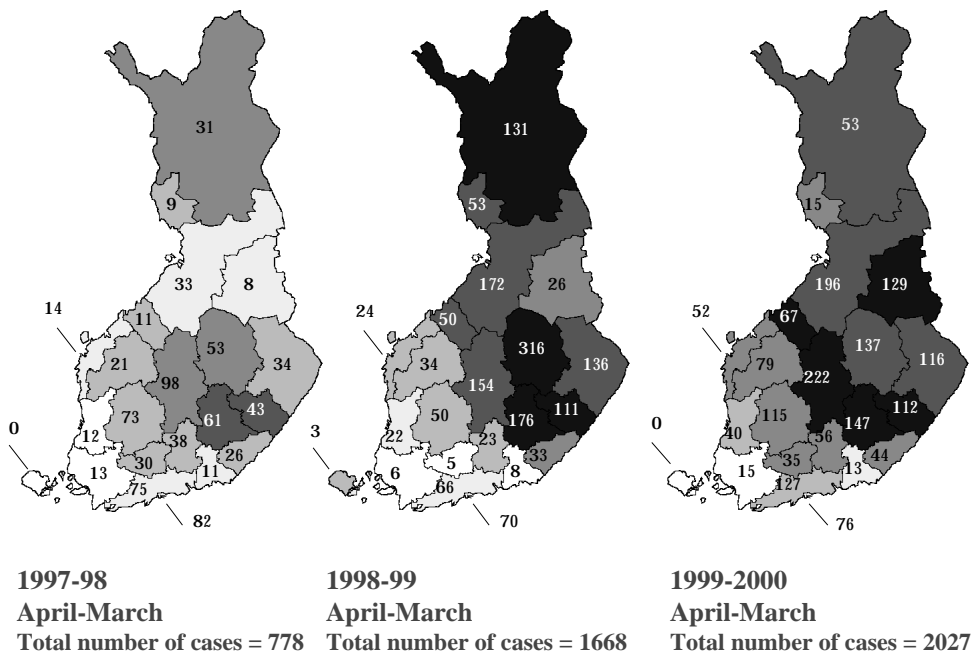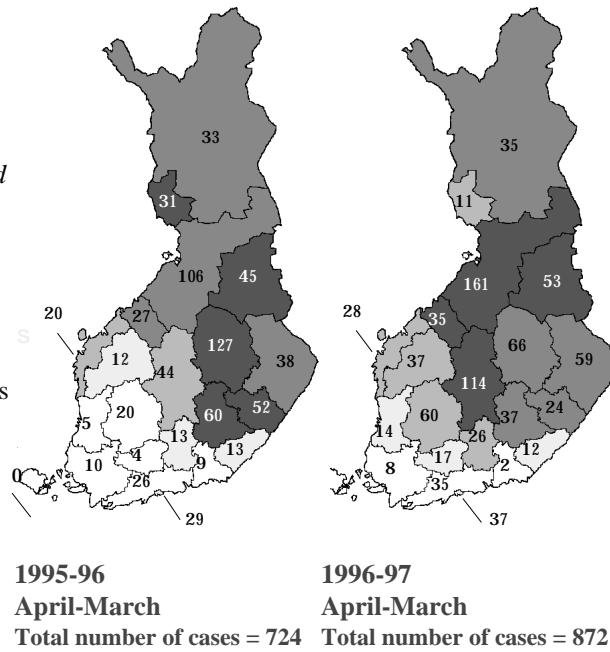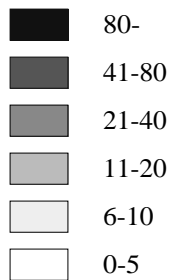

Figure 3. *Monthly distribution of NE cases diagnosed in Finland in 1995-1999.*

Source: National Register of Comminicable Diseases, KTL, Finland, 2000

Figure 4.
Incidence of PUUV infections in each hospital district and including the Åland islands in Finland. The incidences are calculated as cases per 100 000 inhabitants. The number of diagnosed cases in each district is indicated.

Incidence/100 000 inhabitants

⬛	80-
⬛	41-80
⬛	21-40
⬛	11-20
⬜	6-10
⬜	0-5

1995-96
April-March
Total number of cases = 724

1996-97
April-March
Total number of cases = 872

1997-98
April-March
Total number of cases = 778

1998-99
April-March
Total number of cases = 1668

1999-2000
April-March
Total number of cases = 2027

Source: National Register of Communicable Diseases, KTL, Finland, 2000

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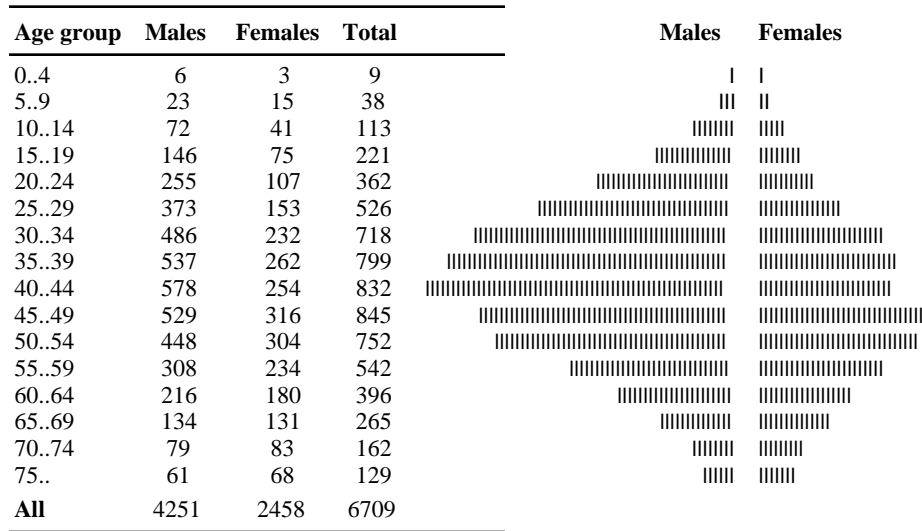| Age group	Males	Females	Total		Males	Females
0..4	6	3	9		I	I
5..9	23	15	38		III	II
10..14	72	41	113		IIIIIIII	IIIII
15..19	146	75	221		IIIIIIIIIIIIII	IIIIIIII
20..24	255	107	362		IIIIIIIIIIIIIIIIIIIIIII	IIIIIIIIII
25..29	373	153	526		IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII	IIIIIIIIIIIIII
30..34	486	232	718		IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII	IIIIIIIIIIIIIIIIIIIII
35..39	537	262	799		IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII	IIIIIIIIIIIIIIIIIIIIIIII
40..44	578	254	832		IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII	IIIIIIIIIIIIIIIIIIIIIII
45..49	529	316	845		IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII	IIIIIIIIIIIIIIIIIIIIIIIIIIIII
50..54	448	304	752		IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII	IIIIIIIIIIIIIIIIIIIIIIIIIIII
55..59	308	234	542		IIIIIIIIIIIIIIIIIIIIIIIIIIII	IIIIIIIIIIIIIIIIIIIIII
60..64	216	180	396		IIIIIIIIIIIIIIIIIII	IIIIIIIIIIIIIIIII
65..69	134	131	265		IIIIIIIIIIII	IIIIIIIIIIII
70..74	79	83	162		IIIIIIII	IIIIIIII
75..	61	68	129		IIIIII	IIIIIII
All	**4251**	**2458**	**6709**			

Figure 5. *Age distribution of NE patients with diagnosed PUUV infection in Finland (1.1. 1995-22.9.2000).* Source: National Register of Communicable Diseases, KTL, Finland, 2000

1.5. Hantaviral diseases

Hantaviruses cause two human diseases: HFRS and HPS. NE caused by PUUV is generally mild form of HFRS, the severity of the disease varying from asymptomatic infection or flu-like illness to infections demanding intensive care, with a mortality of about 0.1% [24]. SEOV causes a moderate form of HFRS, and HTNV and DOBV (in the Balkans), a severe form of HFRS with mortality rates of 4-15%. A connection between the severity of NE and the HLA haplotype of the patient has been demonstrated [107].

The incubation period in HFRS is 2 to 4 weeks, and the disease usually starts with fever and headache, followed by gastrointestinal symptoms (nausea, vomiting, diarrhea), abdominal pain, myalgia and back pain, and, in most cases, by clinical evidence of nephritis with varying degrees of renal insufficiency [75, 151]. In PUUV infection, the symptoms are generally milder and severe complications, such as hemorrhages, are rarer than in DOBV or HTNV infections [6, 15, 75, 120, 139] (Table 4). SNV, ANDV, and related viruses found in the Americas are the causative agents of HPS (Table 3b), a severe acute respiratory distress syndrome [38, 168] with a mortality of about 40%.

Ribavirin [27] lowered the mortality of HFRS in a controlled clinical trial carried out in China

[66]. However, in HPS, no beneficial effects of its use were observed [29]. The treatment of HFRS or HPS patients is thus mainly supportive and is based on understanding of the pathophysiology of the disease and on the evaluation of clinical and laboratory findings.

In some cases, serology can be used as a prognostic marker. The presence of neutralizing antibodies in HPS-patients indicated better survival from SNV infection [21].

Table 4. Clinical characteristics of HFRS

Symptoms and findings	NE*		KHF*	DOBV infection**
	% of patients	Days after onset of fever	% of patients	% of patients
Fever	100	1-7	100	84-100
Headache	90	2-8	86	50-100
Nausea	70	3-7	82	71-79
Stomachache and backache	70	3-9	90	59-85
Oliguria	50	2-8	67	47-100
Polyuria	97	7-14	92	
Increased S-creatinine level	94	2-14	97	95
Proteinuria	94	3-8	100	100
Hematuria	58	3-8	85	100
Dialysis treatment	6		40	19-47
Petechiae	10	3-9	95	59
Hypotension, shock	<10	3-5	40	21-59

* [75]; ** [6, 15, 121, 140]

1.6. Immune response in hantaviral infections

The induction of protective humoral immunity to hantaviruses in humans is believed to be mostly due to viral glycoproteins, since virus-neutralizing activity has been shown to be connected to MAbs raised against the glycoproteins, but not against the nucleocapsid protein. Passive transfer of immune sera or MAbs against glycoproteins before challenge with HTNV protected experimentally infected animals against infection [9, 10, 34, 97, 132, 170].

In humans, IgM, IgG, and IgA antibodies against hantavirus nucleocapsid protein appear soon after onset of symptoms [35, 40, 96, 118] [III]. The IgM antibodies remain detectable for only 1-3 months, whereas the IgG antibodies, and in some patients also IgA antibodies, persist for decades [35, 40, 96, 118] [III].

1.7. Laboratory diagnosis

Serology is the best method to confirm clinically suspected hantavirus infection. Initially, the laboratory diagnosis of acute infection was based on IgG seroconversion in paired serum samples using lung sections of infected rodents or virus-infected cell cultures as IFA antigen. IgM-IFA tests based on cultured cells have also been used [43, 138]. To overcome the specificity problems connected to IgM-IFA, and to avoid the need of collecting paired sera, an IgG-avidity IFA was developed [58]. In this avidity assay, which is based on the maturation of the affinity of antibodies against viral proteins during the course of infection, weak affinity is characteristic of early phase sera, and strong affinity, of late phase sera [58]. Another single-serum test using IgG-IFA (discovered by M. Brummer-Korvenkontio) is based on the fluorescent pattern of the sera in IFA; a granular pattern has been shown to be typical of serum samples collected during the early phase of immunity, and a diffuse pattern, to be typical of serum samples collected during the late phase of immunity [156].

Also, EIAs using cell culture-grown hantaviruses as antigen have been developed [114]. Since hantaviruses are highly pathogenic and grow to low titers in cell culture (demanding at least Biosafety level 3 laboratory facilities), production of antigen for such antibody tests is difficult. Therefore, multiple attempts have been made to produce hantavirus antigens by recombinant DNA technology. Recombinant nucleocapsid proteins have been found suitable, as antibodies to N appear regularly early after onset of symptoms. Bacterial [39, 41, 47, 55, 118, 172, 174, 175] [I], insect [26, 105, 130, 136, 159], and mammalian [47] expression systems have been used for antigen production. Different EIA formats have been introduced, including direct coating-based procedures and various types of capture assays for both IgG and IgM antibodies.

In addition, immunoblotting assays based on recombinant hantavirus N or glycoproteins, or peptides have been presented [47, 60, 71, 173]. For reliable serotyping, i.e. to distinguish between e.g. DOBV and HTNV infections, focus- or plaque-reduction neutralization tests (FRNT or PRNT) [7, 78, 133] run on convalescent phase serum samples are needed due to the high serological cross-reactivity between hantaviruses [94].

For genetic characterization of hantaviruses, several reverse transcriptase (RT)-PCR protocols for detection of hantaviral RNA have been introduced [5, 56, 62, 65, 113, 124, 137, 149]. Sequencing of the amplified regions makes identification of the causative agents feasible, which is of special interest when epidemiological research is conducted in an area where several closely related viruses co-circulate or when new hantaviruses are identified. However, for

routine diagnostics RT-PCR is too insensitive, especially in the case of PUUV infections [124]. Virus isolation from human samples is tedious, and has very limited value for diagnostics, as only a few successful isolations from patients have been obtained [72, 161].

1.8. Protective immunity and vaccines against hantaviral infections

Several attempts have been made to develop a vaccine against hantaviral infections. In addition to traditional vaccines based on inactivated viruses, both recombinant N and glycoproteins, produced in bacterial [152, 153] [II], insect [132, 167] [II], or mammalian [32, 132, 162] expression systems, have been used in protection studies utilizing rodent models. Both N [II] and glycoproteins induce protective immunity, although in some studies, glycoproteins were found to be crucial, and only partial protection was obtained with N alone [132, 162]. Furthermore, studies on DNA vaccination against SEOV [63, 74] and SNV infection [20] have been carried out in laboratory rodents, and shown to have efficacy.

The protective effect of passively transferred antibodies against hantaviruses has been demonstrated in several animal experiments [10, 87, 170] designed to evaluate the therapeutic potential of neutralizing hyperimmune sera or MAbs in post-exposure prophylaxis of hantaviral infections.

A formalin-inactivated HTNV vaccine (Hantavax™) has been commercially available in Korea since 1990. In human vaccine trials, booster vaccinations have been critical for maintaining the antibody levels for more than one year (antibody levels up to 94-100% of persons vaccinated); neutralizing antibody responses have, however, remained at only 50% level of vaccinees [31]. Several other human vaccine trials have been carried out using either inactivated [90, 143, 171] or recombinant vaccines [102]. The vaccines were shown to be safe and well tolerated in healthy volunteers, and capable of eliciting seroresponses in the vaccinees. Booster doses were, however, found to be crucial in order to maintain the antibody levels and potential protection is still unknown.

2. Aims of the study

The aims of the present study were:

1. to develop safer and more efficient ways for hantaviral antigen production
2. to develop and evaluate EIAs for hantavirus antibody detection in human infections caused by European hantaviruses
3. to study the kinetics of human antibody response in hantaviral infections
4. to study the antigenic properties of PUUV-N and its role in protection against PUUV infection in rodent hosts

3. Material and Methods

For detailed descriptions of Material and Methods used in this study, refer to the orignal article(s) identified by Roman numeral(s).

Methods	Used in
Preparative agarose gel electrophoresis	I
SDS-PAGE	I, II, IV, V
Immunoblotting	I, II, IV, V
Enzyme immunoassay (EIA)	I, II, III, IV
Immunofluorescent assay (IFA)	I, II, III, IV, V
Virus cultivation	II
Polymerase chain reaction (PCR)	II, IV, V
Epitope mapping (PEPSCAN)	II
Animal immunization	II
Virus challenge	II
Focus reduction neutralization test	II, IV, V
Bacterial transformation	II, IV, V
Transfection	IV, V
Electroelution	IV
Immunoprecipitation	V

Monoclonal antibodies	Source/[Reference]	Used in
Anti-b-galactosidase	Boehringer	I
Golgi zone	Calbiochem	V
PUUV-N specific MAbs		
1C12	[93]	III, IV
4C3, 3E11, 3G5, 2E12	[93]	IV
PUUV-G specific MAbs		
G1-1E7-1E5	[95]	II
1C9	[98]	II
5B7	[97]	IV
HTNV-N specific MAbs		
G6, F23a1, E5	[166]	IV
TUL-N specific MAb		
1C8	[99]	IV

Expression vectors	Manufacturer/[Reference]	Used in
pEX2	[146]	I
pGEX-2T	Pharmacia	II, IV
pAcYML1	Gibco BRL, [136, 159]	IV
pFASTBAC1,	Gibco BRL	IV
ELVS 2.5	Chiron	V

Recombinant proteins	Expression vector	Used in
β-gal-PUUV-N	pEX2	I
PUUV-rN-1a, -3, -2b, -2c, 2/3	pGEX-2T	II
PUUV-rN-1b (PUUV-1b-GST)	pGEX-2T	II, IV
TUL-rN-Tot, -Eco	pGEX-2T	II
Bac-PUUV-N	pAcYML1	III, IV
Bac-DOBV-N	pFASTBAC1	IV
Bac-HTNV-N	pAcYML1	IV
DOBV-dN-GST	pGEX-2T	IV
r-PUUV-G1, -G2, -N	pELVS 2.5	V

Conjugates	Manufacturer/[Reference]	Used in
Aphos-anti-human IgG	Orion Diagnostica	I
Aphos-anti-human IgM	-"-	I
Aphos-goat anti-mouse IgG	-"-	II
Aphos-donkey anti-mouse IgG	Jackson	II
Perox-streptavidin	Sigma	II
FITC-anti-human IgG	Kallestaad	III
Perox-anti-human IgM	DAKO	III, IV
Perox-MAb 1C12	[93]	III, IV
Perox-anti-human IgG	DAKO	III
Perox-anti-mouse IgG	DAKO	IV
Perox-anti-human IgG	Cappel	IV

Substrates	Manufacturer	Used in
pNPP	Sigma	I, II
DAB	Sigma	II
TMB	Sigma	II, III, IV
OPD	Sigma	III

4. Results and Discussion

4.1. Development and evaluation of antibody tests

4.1.1. PUUV antibody tests based on recombinant antigens

The IgG-IFA in conjunction with the IgG-avidity-IFA [58] were considered reference tests for all recombinant IgG and IgM EIAs developed, since these IFAs could provide a diagnosis of acute infection from a single serum sample, and were the only tests routinely used in our diagnostic laboratory. Because the IgM-IFA had been found to have more specificity problems than the IgG-IFA (data not shown), the IgM-EIAs were not compared with IgM-IFA.

4.1.1.1. Bacterial expression system (I)

Initially, a bacterial expression system with pEX2 vector was used for production of recombinant PUUV-N as a fusion protein with β-galactosidase (β-gal) [157]. The large size of the fusion protein made purification by gel electrophoresis possible. According to immunoblotting analyses using patient sera and rabbit antisera raised against the β-gal -PUUV-N fusion [157], the β-gal -PUUV-N proved to be suitable for use as a diagnostic antigen. Three EIA tests based on β-gal -PUUV-N were developed, one measuring IgM antibodies, one IgG antibodies, and one the avidity of IgG antibodies.

The IgG-EIA correlated well with the reference IgG-IFA. The sensitivity and specificity values of the IgG-EIA were 97.8% and 98.5%, respectively, suggesting that it is an excellent method for diagnostic purposes and for screening of human sera in seroprevalence studies.

Also, the PUUV IgG-avidity EIA showed a good correlation with the IgG-avidity IFA (Fig. 6). With a cut-off value of 20% (ratio of absorbance values of urea-washed and control wells), all old-immunity sera gave in IgG-avidity EIA results indicative of old immunity (>20%). Likewise, all of the acute-phase sera gave results below 20%, indicating acute immunity.

The IgM-EIA gave positive results for a few very early sera, in which no antibodies could yet be detected by IgG-EIA or IgG-IFA, but where subsequent samples confirmed the diagnosis
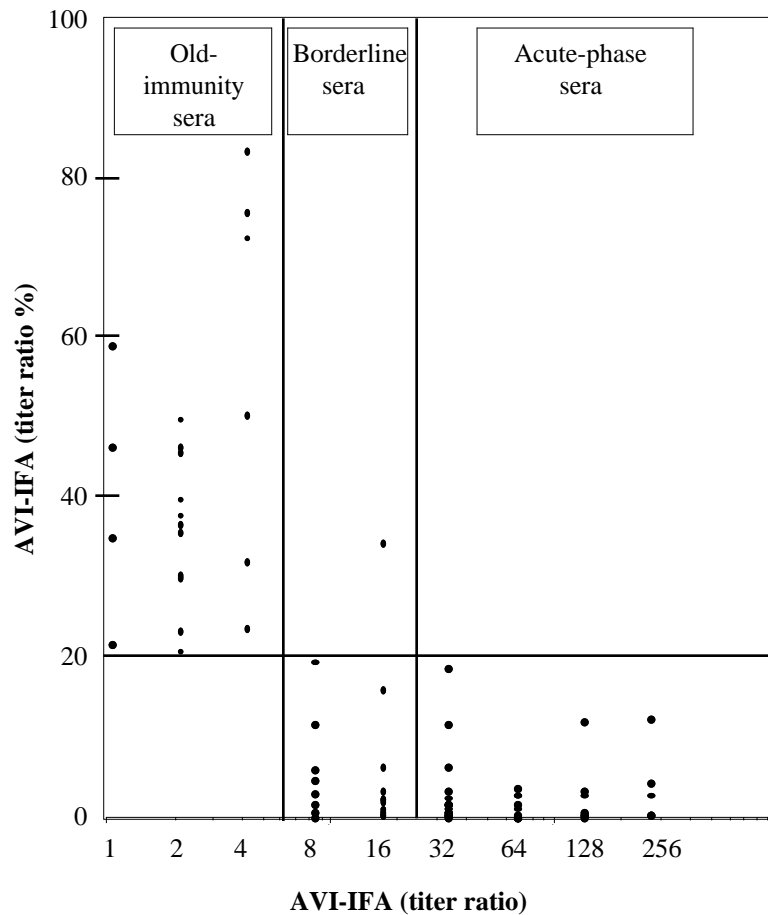
Figure 6. *Correlation between b-gal-PUUV-N IgG AVI-EIA and native PUUV IgG AVI-IFA..*

by IgG seroconversion. The indirect IgM-EIA seemed, however, to give false-positive results among some of the old-immunity sera.

The possible cross-reactivity between hantaviruses was examined using a panel of KHF-patient sera. Approximately one third of the KHF-patient sera showed some reactivity in β-gal-PUUV-N EIA tests as well as in the immunoblotting assay based on the β-gal-PUUV-N antigen. Due to the low cross-reactivity level, the EIA tests based on β-gal-PUUV-N cannot be recommended for the diagnosis of HTNV infections.

4.1.1.2. Baculovirus expression system (III)

A more advanced expression system was introduced for production of recombinant PUUV-N to increase the expression level of the antigen and to avoid the unspecific reactivities caused by the bacterial background in diagnostic tests. The antigenic properties of recombinant baculovirus-expressed PUUV nucleocapsid protein (bac-PUUV-N) were initially shown to be as suitable for use as a diagnostic antigen as native PUUV [25, 70, 159]. The advantage of bac-PUUV-N as compared with b-gal-PUUV-N was demonstrated by the reactivities of a panel of MAbs against the two recombinant proteins. Bac-PUUV-N was recognized by all of the MAbs used, whereas two N epitopes were not found in β-gal-PUUV-N [159]. The diagnostic value of bac-PUUV-N was further evaluated using different assay formats, and the kinetics of the antibody responses studied (III).

After expression in Sf9 insect cells, the insoluble bac-PUUV-N was solubilized by 6 M urea, and run through a Sephadex G20 column. Due to the high expression level of the antigen, the signal-noise ratio was sufficiently high to eliminate background problems in EIAs. Five different EIA test formats were studied: direct-IgM and –IgG EIAs, μ-capture IgM-EIA, Ag-capture IgG-EIA, and IgG-avidity EIA (Fig. 7). The serum panel included sera from NE-patients where the first serum sample had been negative for PUUV IFA, but the second sample indicated a seroconversion. Among routine diagnostic samples, such sera normally comprise 1-5% of all acute cases, whereas in this panel the proportion of such cases was 35%. Using this selected serum panel, the possible differences in sensitivities between the IgG-IFA and different EIAs, especially in the early diagnosis of NE, could be evaluated.
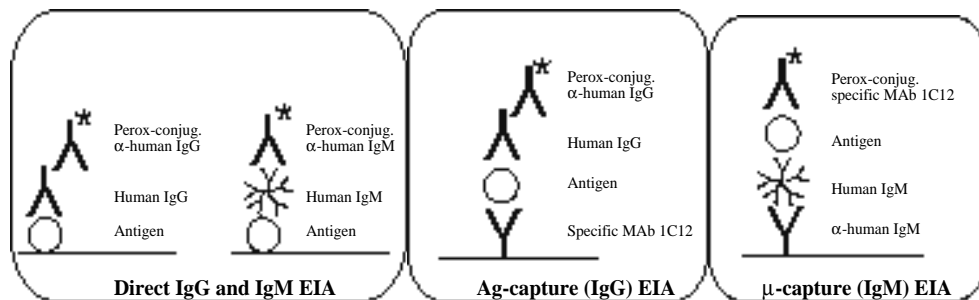

Figure 7. Schematic presentation of the princeples of the different EIA formats used.

As compared with the reference IgG-IFA, the IgG-EIAs were found to be specific, since no false-positive reactions were seen when 90 paired negative sera were tested. Certain differences between the EIAs and IFA could be detected in the sensitivity (Fig. 8a), direct-IgG EIA being the most sensitive of the three IgG assays. Direct PUUV-IgG EIA was found to be more sensitive

for the very early sera (taken on the third day after onset of symptoms or earlier) (Fig. 8a) than capture PUUV-IgG EIA, whereas for older immunity sera the sensitivities were equal. The difference between the two EIAs using the same recombinant antigen was likely to be due to coating the antigen either directly or in a capture format through MAb 1C12, which might occupy or shield some of the aminoterminal "early response" epitopes on bac-PUUV-N. Possible complex formation of bac-PUUV-N might overcome most of this effect, but in some cases, the "shielding effect" may be the reason for the negative results in the early sera with IgG MAb-capture assay.
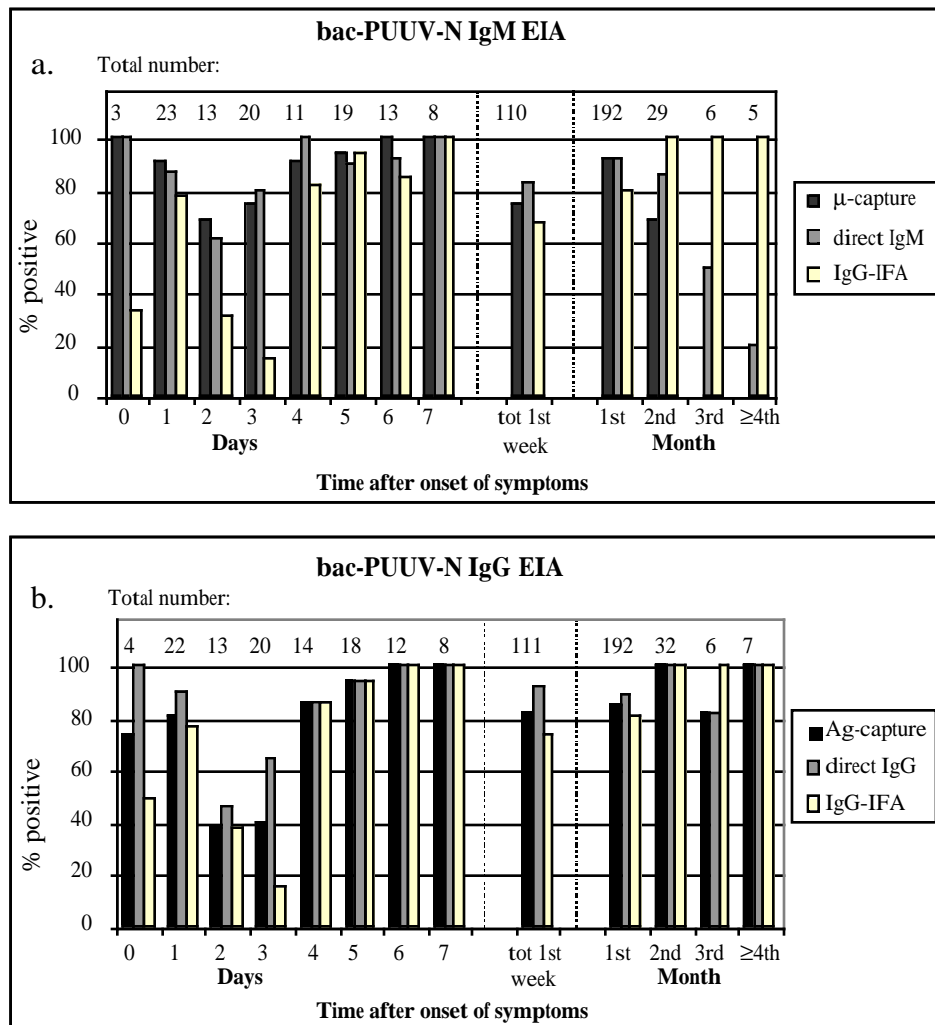
Figure 8 *Comparison of different formats of bac-PUUV-N EIAs.*

The IgG-avidity assay could detect maturation of IgG molecules in serial sera, making it possible to differentiate between old-immunity and acute-immunity sera. The test looked promising as a confirmatory tool for timing IgG responses, although a larger panel of old-immunity sera would be needed to verify the specificity of the low-avidity results.

The two IgM tests were also evaluated with the positive- and negative-control panels. All of the patients became IgM-positive within seven days after onset of symptoms by EIAs (Fig. 8b). Of NE-patient sera taken 0-7 days after onset, 88% (97/110) were positive with μ-capture EIA, 86% (95/110) with direct IgM-EIA, and 65% (72/110) with IgG-IFA.

4.1.1.3. Kinetics of PUUV-N IgM response (III)

Of the bac-PUUV-N EIAs, especially the μ-capture IgM assay seems to be highly suitable for diagnostics as a single test. Its special advantage is that diagnosis can be made very early after onset of illness, even though it should be remembered that a few (probably 1-5%) NE patients can be IgM-negative even 5 days after onset of symptoms (Fig. 8b), in which case, a second serum sample should be taken to confirm the initially negative result. Interestingly, the antibody positivity of the samples taken during the first days (0 and 1 st) after onset of symptoms is close to 100%, declining during the 2nd and 3 rd days, and then rising again to almost 100% (Fig. 8b). This phenomenon might be random variation due to small sample sizes during the first days, or due to differences in the severity of the illness in individual patients. The immunological response may be stronger among those patients seeking medical care earlier than among those who enter the health care system a few days later.

During the first month after onset of symptoms, rate of IgM-antibody positivity started to decline. The number of old sera tested was quite small, but by using the μ-capture assay, the IgM levels declined earlier than by direct-IgM assay (Fig. 8b). The IgG-blocking reagent did not have a significant effect on the IgM results of rheumatoid factor (RF)- positive sera.

4.1.2. Development of DOBV antibody tests based on recombinant antigen (IV)

In our study (IV), DOBV Saaremaa strain cDNA (virus isolated from *A. agrarius* from Estonia) [111] was used as a template to express DOBV-N in bacterial and insect cell expression systems

using pGEX-2T and Bac-to-Bac™ vectors, respectively. The antigens produced were used to develop IgM and IgG EIAs, and to compare the usefulness of different test formats and recombinant proteins in diagnosis of DOBV infection. In addition, tests based on recombinant PUUV-N (bac-PUUV-N and PUUV-1b-N) and HTNV-N (bac-HTNV-N) [136] were included in the comparison.

So far, mainly truncated recombinant antigens, such as either recombinant DOBV-N (aa 1-117) [41] or HTNV-N (aa 1-117 or aa 1-119) [41, 54] have been used in assays to diagnose DOBV infections in Europe. Although our truncated construct (DOBV-DN-GST, aa 1-165) was longer than the previously described ones, the IgM assay as well as the IgG assay based on the truncated antigen failed to detect DOBV antibodies in several sera, and the full-length protein (bac-DOBV-N) was shown to be a more sensitive antigen (Fig. 9). Using a large panel of MAbs, the antigenic properties of bac-DOBV-N were shown to be identical to those of native DOBV-N, although the absence of MAbs raised specifically against DOBV prevented a more complete antigenic evaluation (Table 5).

DOBV IgM-EIAs

DOBV IgG-EIAs
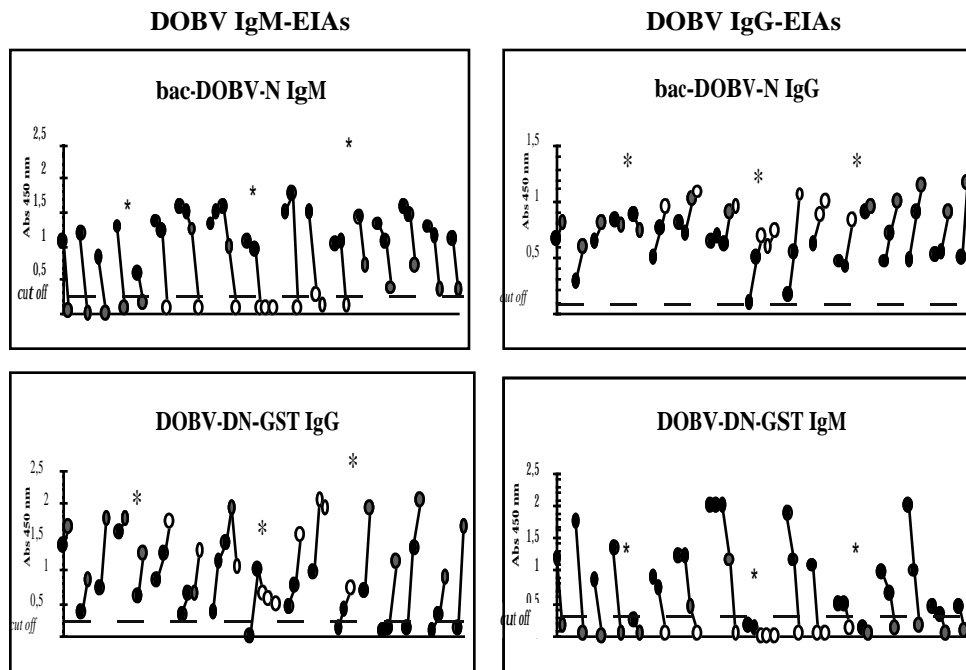
Figure 9. *Comparison of recombinant DOBV-N EIAs.* ● *= acute phase;* ◕ *= convalescent immunity;* ○ *= old immunity; * patients whose diagnosis would have been missed with DOB-DN-GST IgM-EIA alone*

Table 5. *MAb reactivity of recombinant proteins, measured by EIA*

MAb	MAb recognition site (epitope specificity)*	Recombinant antigen (aa)				Native antigen	
		bac-DOBV-N (aa1-429)	DOBV-dN-GST (aa 1-165)	bac-HTNV-N (aa 1-430)	bac-PUUV-N (aa 1-430)	DOBV-IFA	PUUV-IFA
1C12	PUUV-N, aa 1-79 (N-f)	+	+	+	+	+	+
4C3	PUUV-N, aa 1-79 (N-h)	+	+	+	+	+	+
3 E 11	PUUV-N, aa 1-79 (N-f)	+	+	+	+	+	+
3G5	PUUV-N, aa 1-79 (N-d)	-	-	-	+	-	+
2 E 12	PUUV-N, aa 1-79 (N-g)	+	+	+	+	+w	+
5B7	PUUV-G2 (G2-b)	-	-	-	-	-	+
G6	HTNV-N, aa 166-176	+	-	+	+	+	+
F23A1	HTNV-N, aa 205-402	+	-	+	+	+	+
E 5	HTNV-N, aa 166-175	+	-	+	+	+	+
C16D11	HTNV-N, aa 244-429	+	-	+	+	+	+
1C8	TULV-N, aa 1-79	-	-	-	+	-	+

*From Table 9 and [166]; w= weak reaction

One of our aims was to evaluate the suitability of EIAs based on other hantavirus antigens (mainly HTNV and PUUV) for diagnosing DOBV cases in European laboratories. The evaluation was performed on a DOBV serum panel that had been fully characterized and DOBV-specific reactions were confirmed by cross-FRNT using several hantaviruses and also including convalescent sera of the patients. In our study, bac-HTNV-N was found to have nearly the same ability to detect DOBV antibodies as bac-DOBV-N (Tables 6 and 7). This result is in line with the high serological cross-reactivity within the HTNV/DOBV/SEOV group, and indicates that the full-length HTNV-N antigen is generally appropriate for detection of DOBV antibodies. However, recent data have shown that EIAs based on HTNV antigen may in some cases fail to detect DOBV-specific antibodies, both in acute-phase and convalescent samples of HFRS patients [141]. Bac-DOBV-N was not as sensitive in the detection of HTNV-specific antibodies as bac-HTNV-N (Tables 6 and 7), which further supports the concept that homologous antigens are preferable for reliable diagnosis of HFRS. The comparison results of bac-DOBV-N and bac-HTNV-N may, however, have been affected by the expression levels: the expression level of bac-DOBV-N was significantly lower than that of baculovirus-expressed HTNV-N and PUUV-N, possibly due to the different baculovirus expression system (Bac-to-Bac™) used. Despite the low expression level of bac-DOBV-N, the need for purification of the antigen could efficiently be circumvented by the use of the MAb- or μ-capture format in the EIAs. Recent data from our laboratory show that DOBV-N can be efficiently expressed at high levels as a GST fusion protein in insect cells (Koistinen et al., unpublished results).

Table 6a. *Results of IgM-EIAs based on different hantavirus antigens*

Antigen	Negat. sera (N=90)		DOBV-pos. sera (N=60) (acute n=28)		(conv./old n=32)	
	Pos.	Specif. %	Pos. (high/gray-zone)	Sensit. %	Pos. (high/gray-zone)	%
bac-DOBV-N	0	100	28 (28/0)	100	10* (7/3)	31
DOBV-dN-GST	1	99	24 (22/2)	86	4** (3/1)	13
bac-HTNV-N	0	100	28 (28/0)	100	8* (5/3)	25
bac-PUUV-N	0	100	8 (1/7)	29	1* (0/1)	3

*All positive sera are convalescent samples (taken between 1 and 3 months after onset of symptoms);
** Three positive sera are convalescent samples, and one positive serum old-immunity sample (taken later than three months after onset of symptoms)

Table 6b. *Results of IgM-EIAs based on different hantavirus antigens*

Antigen	HTNV-pos. sera (N=20) (acute)		PUUV-pos. sera (N=23) (acute n=18)		(conv./old n=5)	
	Pos. (high/gray-zone)	Sensit. %	Pos. (high/gray-zone)	Sensit. %	Pos. (high/gray-zone)	%
bac-DOBV-N	20 (14/6)	100	2 (0/2)	11	0	0
DOBV-dN-GST	18 (17/1)	90	5 (2/3)	28	1* (1/0)	25
bac-HTNV-N	20 (20/0)	100	5 (3/2)	28	0	0
bac-PUUV-N	8 (3/5)	40	18 (16/2)	100	0	0

Table 7a. *Results of IgG EIAs based on different hantavirus antigens*

Antigen	Negative sera (N=90)		DOBV-pos. sera (N=60) (acute n=28)	(conv./old n=32)	Total
	Pos.	Specif. %	Pos. (high/gray-zone)	Pos. (high/gray-zone)	Sensit. %
bac-DOB-N	0	100	27 (26/1)	32 (32/0)	98
DOB-dN-GST	0	100	21 (19/2)	32 (32/0)	88
bac-HTN-N	0	100	27 (24/3)	32 (32/0)	98
bac-PUU-N	0	100	21 (16/5)	27 (18/9)	80
PUU-1b-GST	1	99	23 (12/11)	22 (11/11)	75

Table 7b. *Results of IgG EIAs based on different hantavirus antigens*

Antigen	HTNV-pos. sera (N=20) (acute)		PUUV-pos. sera (N=23) (acute n=18)	(conv./old n=5)	Total
	Pos. (high/gray-zone)	Sensit. %	Pos. (high/gray-zone)	Pos. (high/gray-zone)	Sensit. %
bac-DOB-N	15 (14/1)	70	4 (1/3)	3 (1/2)	30
DOB-dN-GST	13 (8/5)	65	2 (0/2)	2 (0/2)	17
bac-HTN-N	20 (18/2)	100	7 (7/0)	4 (1/3)	48
bac-PUU-N	5 (1/4)	25	18 (18/0)	5 (5/0)	100
PUU-1b-GST	9 (3/6)	45	22 (21/1)	5 (5/0)	96

4.1.3. Hantavirus diagnostics: general aspects

The earlier reports, later unconfirmed, on HTNV and SEOV infections in Europe are examples of the difficulties in hantavirus typing based on serology using IFA, EIA, or immunoblotting methods. It now appears that the only reliable test for distinguishing the antibody responses against different closely related hantaviruses is the neutralization test. Notably, in these tests the maturation of antibodies is crucial for correct typing: very early sera cannot be used due to cross-reactivity [94] probably caused by IgM-class antibodies. For diagnostic purposes, however, it is most important that the tests sensitively detect early seroresponses against the local hantavirus(es), and although the distinction between e.g. closely related viruses can be achieved in some cases with peptide and truncated recombinant protein based assays, this usually causes a loss of sensitivity [71, 105] (Araki et al., unpublished results)..

Serological assays are needed for hantavirus diagnostics, since only about 67% of PUUV patients [124] and about 40% of DOBV patients [119] have been shown to be hantavirus RNA-positive by RT-PCR, and the isolation of hantaviruses from HFRS patients is rarely successful [72, 161]. For serological diagnosis of hantaviral infections, the assays measuring IgM antibodies are the method of choice as the IgM levels rise earlier than those of IgG antibodies, and the IgM antibody response is definitively associated with acute infection [25, 43] [III]. Yet, it should be noted that the format of the assay has a major impact on its specificity and sensitivity. If the expression level of an antigen is low, the use of a capture format may minimize the need for purification of the antigen. On the other hand, the less than full length of the recombinant protein can affect its capacity to bind antibodies to different epitopes and may thus lower the sensitivity if capture assays are used [25].

Currently, a few diagnostic kits are also commercially available for hantavirus serology. Progen (Heidelberg, Germany) has provided PUUV and HTNV EIAs for detection of IgM and IgG antibodies. The tests are based on truncated recombinant nucleocapsid proteins. However, according to an international evaluation, these PUUV-specific IgM and IgG kits were found to be of lower sensitivity than FRNT, PUUV-IFA, or in-house EIAs based on bac-PUUV-N, *E. coli*-expressed PUUV-DN, or native PUUV [25]. Furthermore, MRL Diagnostics (CA, USA) has recently introduced EIAs for detection of hantavirus IgM and IgG antibodies; the tests are based on a cocktail of baculovirus-expressed recombinant DOBV and SEOV nucleocapsid proteins. IFAs based on Vero E6 cells infected with either PUUV or HTNV have also been provided by Progen. In addition, a rapid PUUV IgM test using immunochromatography has been developed based on bac-PUUV-N. The sensitivity and specificity values of this test are 97-100% [67].

The m-capture assay for the early detection of PUUV and DOBV IgM antibodies gave the best results. Furthermore, if a negative result is obtained from a sample taken before the 6th day after onset of symptoms, a second sample is recommended to exclude or confirm hantavirus infection. The level of cross-reactivity between hantaviruses is highest among the HTNV/ DOBV/SEOV group, and within the PUUV/SNV/TUL group, but for accurate diagnosis in geographical areas in which hantaviruses from both groups cocirculate, at least one antigen from each group homologous to local viruses should be used; i.e. in Europe PUUV and DOBV.

4.2. Characterization of PUUV-N (II)

Truncated PUUV-N proteins were produced as GST-fusion proteins in a bacterial expression system using a pGEX-2T vector (Fig. 10). The sequences for the expressed fragments were selected according to possible structural domains and hydrophilic/immunogenic regions from hydrophilicity values and structure predictions. The immunogenicity of truncated recombinant PUUV-N proteins and bac-PUUV-N was analyzed, and an animal model to investigate the role of PUUV-N in protective immunity in the carrier rodent was developed. Unlike in previous experiments using hamsters, the natural host rodent of PUUV, bank vole (*Clethrionomys glareolus*), was used in this study (Fig. 11).
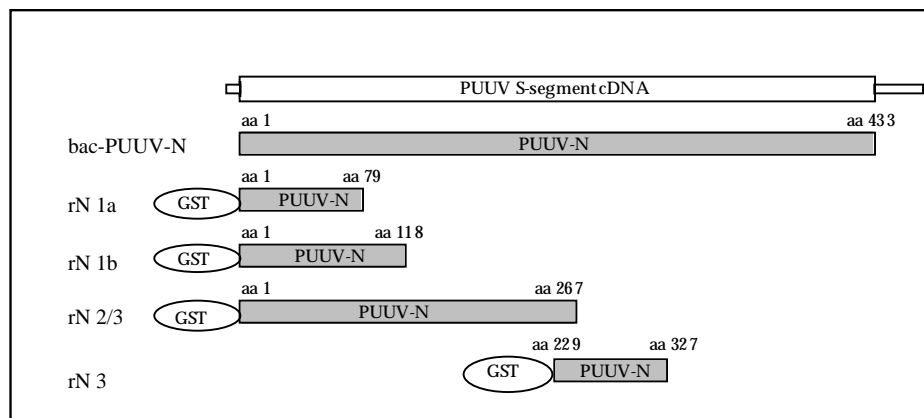
Figure 10. *Recombinant PUUV-N constructs for immunization studies.*
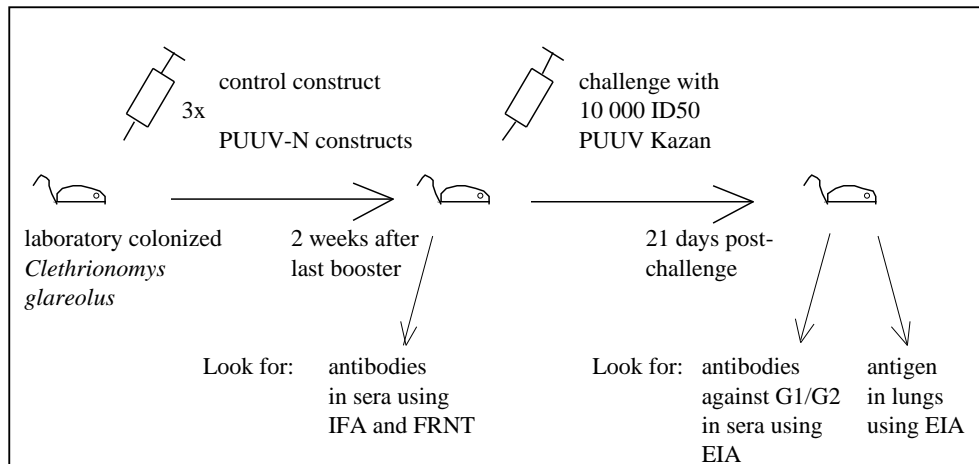
Figure 11. *Schematic representation of the PUUV protection assays in colonized bank voles (Clethrionomys glareolus).*

4.2.1. B-cell epitopes in PUUV-N

When truncated PUUV-N constructs were used for epitope mapping of MAbs generated from a virus-infected bank vole, six of seven epitopes were mapped within the N-terminal 20% of the PUUV-N (aa 1-79), thereby indicating that this part of PUUV-N is a major antigenic region (Table 8). This is in agreement with previous results based on additivity and competitive EIAs, which together with the reactivity patterns with various hantavirus strains suggests that several of the epitopes were partially or completely overlapping [93, 97]. The MAb 3H9 that had been characterized previously [92] was also in this study shown to react with the most variable part of PUUV-N (aa 229-267). Our result is in line with other studies on B-cell epitopes on PUUV-N, TULV-N, and HTNV-N [92, 99, 166].

Polyclonal sera from naturally or experimentally infected bank voles revealed the presence of B-cell epitopes over the entire N. Although sera from infected animals were non-reactive with the rN-2b fragment (aa 135-214), PEPSCAN data indicated the presence of antigenic domains also within this region. Studies on the human IgG response to PUUV-N have shown a similar pattern: truncated N proteins indicated that the amino-terminal part is the major antigenic region, although PEPSCAN data revealed the presence of antigenic domains in other parts of the protein as well [42, 55, 92, 156, 157]. Similarly, for SNV and HTNV, the major domain for the humoral reactivity has been shown to reside within the amino-terminus of *E. coli*-expressed N proteins [71, 163, 166].

Table 8. *Summary of MAb reactivity in immunoblotting with truncated rN proteins*

Antigen	MAb (epitope)						
	3H9 (N-a)	5 E1 (N-b)	5B5 (N-c)	3G5 (N-d)	1C12 (N-f)	2 E12 (N-g)	4 E5 (N-h)
PUUV							
rN 1a (1-79)	-	+	+	+	+	+	+
rN 1b (1-118)	-	+	+	+	+	+	+
rN 2/3 (1-267)	+	+	+	+	+	+	+
rN 3 (229-327)	+	-	-	-	-	-	-
bac-PUUV-N	+	+	+	+	+	+	+
TULV							
rN Eco (1-61)	-	-	-	-	+	-	+
rN Tot (1-430)	-	-	-	(+)	+	+	+
Epitope region (recognized aa's)	229-267	1-79	1-79	61-79	1-61	61-79	1-61

+ positive reaction; (+) weak reaction; - negative

4.2.2. Protective immunity in natural host by immunization with PUUV-N (II)

Examination by IFA revealed that all the different recombinant PUUV-N fragments elicited in animals significant IgG levels reactive with native PUUV-N (Table 9). The highly immunogenic nature of the amino-terminal region was further demonstrated by the relatively high antibody titers to native PUUV-N evoked in animals immunized with rN-1a (aa 1-79); none of the pooled antisera raised to the larger rN fragments or to the entire N (i.e. bac-PUUV-N or during viral infection) showed higher titers to native N (Table 9).

Although HTNV causes systemic infection with lethal outcome in newborn mice [76, 103, 110], nude mice [109], and SCID mice [165], no animal model for HFRS-like disease has been found, making it impossible to evaluate the ability to moderate or prevent disease by immunization with PUUV recombinant proteins. Thus, another experimental approach, based on infection of the natural host, the bank vole, was used for measurement of protection from

Table 9. *Immune responses to PUUV in bank voles after immunization with different recombinant PUUV-N or control constructs, and infection with PUUV*

Immunogen (aa)	N animals	Reciprocal end-point titers		
		IFA Native PUUV	FRNT Native PUUV	G1/G2 EIA Native PUUV
rN 1a (1-79)	2	6400	< 40	< 200
	3	3200	< 40	< 200
rN 1b (1-118)	3	3200	< 40	< 200
rN 2/3 (1-267)	3	1600	40	< 200
rN 3 (229-327)	3	3200	< 40	< 200
bac-PUUV-N (1-433)	2	1600	< 40	< 200
GST-control	5	<100	< 40	< 200
PUUV (wild) a	5	1600	1280	6400
PUUV (Kazan) b	5	1600	1280	12800
Non-immune control	2	<100	< 40	< 200

[a]Sera from PUUV IgG-positive wild bank voles trapped in northern Sweden; [b]Sera drawn 3 weeks after experimental infection with PUUV strain Kazan.

PUUV infection. None of the bank voles immunized with the amino-terminal fragments or with complete recombinant N (bac-PUUV-N) displayed N antigen in their lungs after challenge with infectious PUUV. Animals were protected against challenge virus in up to 10^4 infectious doses. When post-challenge sera of these animals were analyzed, only one (immunized with the shortest aminoterminal fragment (aa 1-79)) had glycoprotein-specific antibodies and appeared not to be fully protected (Table 10). Even though the number of animals in some groups was small, all animals immunized with proteins corresponding to aa 1-118 or with larger amino-terminal fragments of PUUV-N, were well protected against infection. Bank voles have also been shown to be protected against PUUV infection when hepatitis B virus core particles carrying PUUV-N constructs were used in similar protection experiments [152, 153]. In other experimental settings, HTNV recombinant N has been shown to protect hamsters and suckling mice from HTNV infection [132, 167]. The results from our animal model emphasize the importance of investigating not only the presence of viral antigen, but also the antibody responses.

Antibodies against envelope glycoproteins carry best protection in passive transfer of antibodies in experimental models. Also neutralizing activity is detected *in vitro* for MAbs directed to G1 and G2, but not to N [9, 10, 34, 97, 132, 170].

Table 10. *Presence of antigen or G1/G2 antibodies after challenge with PUUV/Kazan in bank voles immunized with recombinant PUUV-N constructs*

Immunogen (aa)	Antigen in lungs	G1/G2 antibody
rN 1a (1-79)	0/5[a]	1/5[b]
rN 1b (1-118)	0/3	0/3
rN 2/3 (1-267)	0/3	0/3
rN 3 (229-327)	1/3	1/3
bac-PUUV-N (1-433)	0/8	0/8
GST-control	5/5	5/5
Non-immune control	8/8	8/8

[a]Number of N-antigen positive/number inoculated; [b]Number of G1/G2-specific antibody positive/number inoculated

The significance of the N-specific antibody response *in vivo* is, however, not yet completely understood. A MAb specific to HTNV-N has been shown to protect from virus infection, and N-specific polyclonal sera, to significantly increase the survival time in a mouse model [167]. N-specific MAbs are known to partially protect bank voles from PUUV infection (Lundkvist et al., unpublished). Accordingly, the humoral response to N may, in addition to the glycoprotein-specific response, be of importance for the immunity, e.g. via antibody-dependent cell-mediated cytotoxicity and/or complement-mediated cytolysis.

Cell-mediated response to HTNV has been shown to be induced in experimental infection in rodents and also to be at least partially protective against infection [11, 12, 167]. Human CD4+ and CD8+ cytotoxic T lymphocyte (CTL) epitopes have been identified on SNV-N (aa 131-139, aa 234-242, and aa 372-380) during the acute phase of HPS [46], and on HTNV-N (aa 12-20 and aa 421-429) years after laboratory-acquired subclinical HTNV infection [154]. T-cell epitopes on HTNV-N (aa 221-228, aa 328-335, and aa 422-429) have also been described using a mouse model [121]. Recently, CTL responses against both PUUV G1 and G2 were seen in natural PUUV infection (Van Epps et al., personal communication). Our recombinant proteins, which were found to be protective in bank voles, covered the same PUUV-N regions shown to be important as human T-cell epitopes.

Our data suggest that recombinant PUUV-N proteins are capable of inducing a response that can protect animals from infection after challenge with high doses of infectious virus.

4.3. Expression of PUUV proteins in mammalian cells (V)

To establish a recombinant expression system for the glycoproteins, and to compare the kinetics of human antibody responses towards recombinant PUUV-N, G1, and G2, these proteins were expressed in mammalian BHK-21 cells using an alphavirus replicon system. This expression system was selected to allow proper post-translational processing of the recombinant glycoproteins in mammalian cells, as our previous experience with the baculovirus expression system suggested that the glycoproteins were not properly folded in insect cells [159].

The antigenic properties of the recombinant proteins were evaluated using panels of specific MAbs raised against PUUV-N, G1, or G2. Recombinant PUUV-N and glycoproteins, when expressed together, were found to react with the MAbs reactive against conformational epitopes identically to native viral proteins (Table 11), suggesting proper folding of these recombinant proteins. Co-expression of G1 and G2 was essential, since individually expressed G2 was not recognized by all G2-specific MAbs, and was thus apparently not properly processed. A stronger response against MAbs was obtained when G1 and G2 were simultaneously expressed from separate transcripts as compared with expression from a single open reading frame. Immunoprecipitation by polyclonal rabbit antisera against G1, G2, and N showed that the sizes of the recombinant proteins were similar to those of native viral proteins.

Table 11. *Reactivity of PUUV-specific monoclonal antibodies with the recombinant proteins, as analysed by IFA*

MAb	MAb recognition site (epitope specificity)	Recombinant protein					Native antigen
		G1	G2	M*	G1+G2**	N	PUUV Sotkamo
MAb 5A2	PUUV-G1 (a)	+	-	+w	+	nd	+
MAb 4G2	PUUV-G2 (a1)	-	+	+	+	nd	+
MAb 1C9	PUUV-G2 (a2)	-	+	+	+	nd	+
MAb 5B7	PUUV-G2 (b)	-	-	+w	+	nd	+
MAb 3H9	PUUV-N (a)	nd	nd	nd	nd	+	+
MAb 5E1	PUUV-N (b)	nd	nd	nd	nd	+	+
MAb 5F4	PUUV-N (e)	nd	nd	nd	nd	+	+
MAb 1C12	PUUV-N (f)	nd	nd	nd	nd	+	+
MAb 2E12	PUUV-N (g)	nd	nd	nd	nd	+	+
MAb 4C3	PUUV-N (h)	nd	nd	nd	nd	+	+

*pELVS-PUUV-M- transfected to express G1 and G2 proteins; **pELVS-PUUV-G1 and pELVS-PUUV-G2 transfected simultaneously; w= weak reaction; nd=not determined

Previous studies using vaccinia virus-based expression systems have indicated that HTNV G1 and G2 can be expressed separately, but that the transport of G2 from the endoplasmic reticulum to the Golgi complex is dependent on coexpression with G1. However, the transport of G1 has been debated; it has been found to be targeted to Golgi when expressed alone [122] or to be dependent on coexpression with G2 [131]. Our results on obtaining conformationally properly folded PUUV G1 and G2 by coexpression further support the close interplay between the two glycoproteins in hantaviruses, but whether the dependence of proper folding of G2 on the presence of G1 is associated with Golgi transport awaits further studies. Moreover, hantavirus glycoproteins may differ in their targeting [128], and no thorough localization studies have been done for PUUV.

To evaluate the human IgG-antibody responses against recombinant G1, G2, and N, several panels of sera were tested by IFA. The best reactivity was obtained when G1 and G2 expressed together were used as antigen. Only 2% of the acute-phase sera (N= 133) contained IgG antibodies against PUUV G1+G2, whereas of old-immunity sera (N= 100), 87% were G1+G2-positive (Table 12). These results are in line with previous data obtained by assays based on native structural PUUV proteins [96]. Using a panel of serial patient sera, it was shown that as the immunity matures, IgG antibodies against recombinant glycoproteins appear and finally high titers are reached at late convalescence, while antibodies to nucleocapsid protein are present in high titers already in the acute phase of infection (Table 13).

Table 12. Presence of IgG antibodies to recombinant PUUV-G1 and -G2-antigens in PUUV- and DOBV-patient sera by IFA

Recombinant antigen	PUUV-patient sera				DOBV-patient sera			
	Acute-phase sera (tot=19)		Old-immunity sera (tot=81)		Acute-phase sera (tot=24)		Convalescent- or old-immunity sera (tot=16)	
	N pos.	% pos.	N pos.	% pos.	N pos.	% pos.	N pos.	% pos.
G1	1	5	65	80	nd		nd	
G2	0	0	27	33	nd		nd	
G1+G2*	1	5	70	86	0	0	5	31
M**	0	0	60	74	nd		nd	

*Separate pELVS-PUUV-G1 and pELVS-PUUV-G2 transfected simultaneously; **whole pELVS-PUUV-M segment encoding for G1 and G2 transfected, nd = not determined

Table 13. PUUV antibody kinetics in serially bled NE-patient sera

Patient no.	Days post onset	IFA pattern native PUUV*	EIA results* IgG	EIA results* IgM	IgG-IFA results (recombinant antigens) G1	G2	M	G1+2	titer	N	titer
I	4	g	+	+	-	-	-	-	-	+	2560
	12	dg	+	+	-	-	+w	+w	10	+	40960
II	-	g	+	+	+	-	-	-	-	+	2560
	10	g	+	+	-	-	-	-	-	+	10240
	182	d	+	-	+	+	+	+	5120	+	5120
III	1	-	-	-	-	-	-	-	-	-	-
	23	dg	+	+	+	-	+	+	640	+	20480
	63	d	+	-	+	+	+	+	640	+	10240
IV	4	?+	+	+	-	-	-	-	-	+	1280
	18	dg	+	+	-	-	-	-	-	+	5120
	69	d	+	-	-	-	-	-	-	+	1280
V	30	g	+	+	+	-	+	-	-	+	10240
	60	dg	+	-	+	+	+	+	2560	+	10240
	169	d	+	-	+	+	+	+	10240	+	10240
VI	12	g	+	+	-	-	-	-	-	+	81920
	31	d	+	+	+	-	+	+	640	+	20480
	82	d	+	-	+	+	+	+	5120	+	20480
VII	9	g	+	+	-	-	-	-	-	+	20480
	16	g	+	+	+	-	-	+w	40	+	20480
	118	d	+	-	+	+	+	+	10240	+	20480
	234	d	+	-	+	+	+	+	20480	+	5120
VIII	5	g	+	+	-	-	-	-	-	+	2560
	117	d	+	-	-	-	+w	-	2560	+	5120
IX	14	g	+	+	+	-	-	-	-	+	81920
	868	d	+	-	+	+	+	+	2560	+	5120

g = granular fluorescence pattern (in IgG-IFA); d = diffuse fluorescence pattern (in IgG-IFA); d/g = intermediate fluorescence pattern (in IgG-IFA); w = weak reaction; * bac-PUUV-N; IFA = immunofluorescent assay; EIA = enzyme immunoassay

With a panel of FRNT-verified DOBV sera, the cross-reactivity between the sera and recombinant PUUV G1+G2 antigen was observed to be quite low (36% of the sera showed some reactivity) (Table 12), and the intensity in IFA was weak among the old–immunity sera. Of the acute DOBV sera, none were reactive (Table 12). This result is consistent with the

finding that glycoproteins are the most variable of hantaviral proteins [123]. Whether the comparison of the titers of patient serum antibodies against different hantavirus glycoproteins could be used for typing of the causative agent remains to be seen.

Our results are in contrast to those obtained with early-phase sera of HPS patients, which reacted with bacterially expressed truncated recombinant SNV G1 protein. However, in those experiments, no reactions between acute PUUV sera and SNV G1 were seen [60] (no old-immunity PUUV sera were included in that material). One explanation for the discrepancy could be that the reactivity is due to antibodies against virus-type specific linear epitopes. A similar phenomenon has been documented in human parvovirus infections, in which certain linear epitopes are detectable only in the acute phase of immunity [73, 142].

Sera with IgG antibodies against recombinant N showed in native PUUV IgG-IFA the granular fluorescence pattern associated with the acute phase of immunity [156] (Table 14). Whereas sera with IgG antibodies also against the recombinant glycoproteins showed in native PUUV IgG-IFA the diffuse fluorescence pattern associated with late phase of immunity (Table 14). This further confirms that the native IgG-IFA fluorescence pattern, which has been used in our routine diagnostics as a rapid test to distinguish acute PUUV infections from old PUUV immunity, is highly useful for timing the antibody response. In our panel, a granular fluorescence pattern had a specificity of 100% and a sensitivity of 97% for acute PUUV infection (Table 14), as compared with m-capture IgM EIA. In addition, all cases with a diffuse fluorescence pattern were PUUV IgM-negative (Tables 13 and 14).

Table 14. *Reactivity and fluorescence pattern of sera in native PUUV-IgG-IFA as compared to IgM-EIA and to reactivity with recombinant PUUV-G1 and -G2 antigens in IgG-IFA*

Sera (PUUV)	Total	IgG- IFA (native PUUV) IFA pattern			IgM-EIA bac-PUUV-N	IgG-IFA ELVS-G1+G2
		Positive (%)	Granular (%)	Diffuse (%)	Positive (%)	Positive (%)
Acute-phase	104	101 (97)	101 (97)	0	104 (100)	2 (2)
Old-immunity	9	9 (100)	0	9 (100)	0	8 (89)
Negative	87	0	-	-	0	0

The pELVS constructs can also be applied in DNA vaccination studies. Preliminary evidence suggests that the pELVS-PUUV-N construct provides protection in the bank vole model (Lundkvist et al, unpublished results).

5. Concluding remarks and Future prospects

Hantaviruses include several world-wide distributed human pathogens, transmitted to humans via excreta of infected carrier rodents. These viruses are associated with two clinical diseases, HFRS and HPS, which vary in severity depending on the causative agent. Hantavirus infections are especially common in China, Korea, Russia, and Northern Europe; in Finland alone approximately one thousand cases are diagnosed annually.

This thesis summarizes our results on the production and use of recombinant hantaviral proteins in studies on the antigenic properties of these proteins, characterization of domains involved in protective immunity, and development of diagnostic applications for hantaviral diseases.

For hantavirus diagnostics, serology is the method of choice, because virus isolation is rarely successful, and the value of detecting viral RNA by RT-PCR is limited in practice at least for PUUV. For optimal sensitivity, assays based on antigens from viruses circulating in each geographical region are recommended. In Europe, two hantaviruses causing HFRS, namely PUUV and DOBV, are circulating. Recombinant PUUV and DOBV nucleocapsid proteins were expressed in bacterial or insect cells, and based on these antigens, EIAs were developed to measure IgG and IgM antibody responses in humans. These tests were found to be specific and sensitive for diagnostic use. For diagnosis of acute infection, μ-capture EIA based on baculovirus-expressed full-length nucleocapsid protein (PUUV-N and/or DOBV-N) is recommended. Furthermore, if a negative IgM result is obtained from a sample taken before the 6th day after onset of symptoms, a second sample should be taken to exclude or confirm hantavirus infection.

Co-expression of the recombinant glycoproteins G1 and G2 was found essential for proper post-translational processing of the proteins as evaluated by MAbs against conformational epitopes. IgG antibodies against glycoproteins appeared only in the late convalescent phase, whereas IgG antibodies against N were seen in high titers already in the acute phase of NE-patient sera. The appearance of IgG antibodies to glycoproteins was associated with a diffuse type of fluorescence in native PUUV IgG-IFA; the granular fluorescence due to early anti-N response was shown to be diagnostic for acute infection.

Determination of the type of the causative agent of hantaviral infection is of interest in seroepidemiological and clinical studies. The method of choice at the moment is the neutralization assays performed on convalescent-immunity sera. Attempts have been made to develop other typing methods based on truncated nucleocapsid proteins as antigens in EIA,

and alternative approaches could also be based on the use of recombinant glycoproteins.

Truncated PUUV nucleocapsid proteins were used to map the B-cell epitopes on N by use of MAbs. The aminoterminal part of PUUV-N was shown to be highly immunogenic, and in protection experiments, immunization with recombinant PUUV-N or its aminoterminal fragments (expressed in insect or bacterial cells) were found to be capable of inducing protection against PUUV infection in bank voles. Because a rodent model may not be directly comparable with PUUV infection in man (a dead-end for the virus), our future vaccine prospects will include the use of a primate animal model to further study the suitability of PUUV-N and glycoprotein constructs described in this study as recombinant protein or DNA vaccines.

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