Aus dem Institut für medizinische Virologie
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DISSERTATION

Genetic reassortment between members of different Dobrava-Belgrade virus lineages and allocation of innate immune response modulation to particular genome segments

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von

Sina Kirsanovs

aus Bremen

Gutachter/in: 1. Prof. Dr. med. D. H. Krüger

2. Prof. Dr. med. H.-W. Presber

3. Priv.-Doz. Dr. R. Ulrich

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Abstract

Hantaviruses possess a tri-segmented negative-stranded RNA genome with the potency of genetic reassortment. Reassortment processes between genome segments might cause dramatic changes in the virulence of viruses as has been shown for influenza viruses. The European Dobrava-Belgrade virus species (DOBV) forms distinct lineages associated with different *Apodemus* mice species and can cause hemorrhagic fever with renal syndrome of different clinical severities.

In this study, virological and molecular tools to monitor RNA reassortment in cell culture between two genetic lineages of DOBV were established. Representatives of the DOBV-Af (associated with *A. flavicollis*) and DOBV-Aa (associated with *A. agrarius*) lineages were used for dual infection of Vero E6 cells.

Two hundred and seven individual virus clones were isolated and screened for reassortment by a newly established strain- and segment-specific multiplex PCR (MP-PCR). After co-infection, as much as 31% of virus progeny population was represented by genetically stable reassortants. Reassortment was proven by sequence analyses of the complete S and M segments as well as L-ORF. Two stable reassortment patterns where identified. In all cases, S and L segments originated from the same parental strain and only the M segment was exchanged. Since the different virulence of DOBV-Aa vs. DOBV-Af in humans could be associated with the two viruses´ different abilities to modulate the innate immune response of the host; Three "read-out markers" (physiological role of RIG-I on virus replication, induction of the interferon-stimulated response element and of the antiviral MxA protein) after virus infection were investigated. Clear differences to modulate these parameters of innate immune responses were found between DOBV-Aa on the one hand and DOBV-Af on the other. These two different phenotypes were reflected by the two types of reassortants carrying the respective S and L segments of the parental virus. The differential induction/modulation of innate immune responses might represent an important determinant of (different) virulence of hantaviruses in the human organism.

Zusammenfassung

Hantaviren besitzen ein in drei Segmente gegliedertes, einzelsträngiges RNA Genom in Negativstrangorientierung. Aufgrund der Segmentierung besteht die Möglichkeit des genetischen Reassortment. Genetisches Reassortment zwischen Genomsegmenten verschiedener Viren kann zu dramatischen Veränderungen der Virulenz führen, wie es für Influenzaviren beschrieben ist.

Die europäische Virus Spezies Dobrava-Belgrad (DOBV) bildet, assoziiert mit verschiedenen *Apodemus* Spezies, verschiedene genetische Linien und verursacht hämorrhagisches Fieber mit renalem Syndrom mit verschieden schweren klinischen Verläufen.

In dieser Doktorarbeit wurden virologische und molekularbiologische Methoden etabliert um genetisches Reassortment zwischen zwei genetischen DOBV-Linien in Zellkultur nachzuweisen und zu untersuchen. VeroE6 Zellen wurden mit Vertretern der genetischen Linien DOBV-Af und DOBV-Aa ko-infiziert. Insgesamt wurden 207 Virusklone isoliert und mittels einer neu etablierten stamm- und segmentspezifischen Multiplex-PCR analysiert. Es wurden 31% der isolierten Virusklone als genetisch stabile Reassortanten identifiziert. Das genetische Reassortment wurde durch Sequenzanalysen des kompletten S und M Segments und des kompletten open reading frame des L Segments bestätigt. Es wurden nur zwei stabile Reassortmentmuster gefunden; in beiden Fällen stammten das S and L Segment von dem gleichen Elternstamm, während das M Segment ausgetauscht war. Die unterschiedliche Virulenz von DOBV-Aa gegenüber DOBV-Af im Menschen könnte mit der Fähigkeit beider Viren korrelieren, das angeborene Immunsystem unterschiedlich zu modulieren. Drei „Read Out Marker“, die physiologische Rolle von RIG-I für das Wachstum von Hantaviren, sowie die Aktivierung des „interferon stimulated response Element“ und die Induktion des antiviralen MxA Proteins, wurden untersucht. Anhand dieser Parameter wurden klare Unterschiede in der Modulierung der angeborenen Immunantwort durch DOBV-Aa auf der einen Seite und durch DOBV-Af auf der anderen Seite gefunden. Diese beiden Phänotypen wurden durch die Reassortanten, die das S und L Segment des jeweiligen Elternvirus trugen, bestätigt. Die unterschiedliche Beeinflussung der angeborenen Immunantwort kann eine mögliche Determinante der Virulenz von Hantaviren im menschlichen Organismus aufzeigen.

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Abbreviations

aa	amino acid
Aa	*Apodemus agrarius*
Af	*Apodemus flavicollis*
ANDV	Andes virus
APS	ammonium persulphate
BCCV	Black Creek Canal virus
BME	basal medium with Earls's salt
BSL	biosafety level
CCHFV	Crimean Congo Hemorrhagic Fever Virus
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethylsulfoxide
DNA	deoxyribonucleic acid
DOBV	Dobrava virus
dsRNA	Double stranded RNA
EDTA	ethylene-diamine-tetra-acetic acid
FCS	fetal calf serum
FFU	focus forming unit
HBSS	Hank´s buffered saline solution
HCPS	hantavirus cardiopulmonary syndrome
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
HFRS	hemorrhagic fever with renal syndrome
HRP	horseradish peroxidase
HTNV	Hantaan hantavirus
ICTV	International Committee on Taxonomy of Viruses
IFN	interferon
IFNAR	IFN-α/β receptor
IRF	IFN regulatory factors
ISGF	IFN-stimulated gene factor
ISRE	IFN-stimulated response element
JAK	Janus protein tyrosine kinase
kb	kilo base
kbp	kilobasepairs
L	large genome segment
LACV	LaCrosse virus
M	medium genome segment
MEM	minimum essential medium
ML	Maximum-likelihood
MOI	multiplicity of infection

N	nucleocapsid
N protein	nucleocapsid protein
NE	nephropathia epidemica
NS	non-structural
nt	nucleotides
ORF	Open reading frame
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PFA	paraformaldehyde
PHV	Prospect Hill hantavirus
PKR	protein kinase R
PRR	Pattern recognition receptor
PUUV	Puumala hantavirus
RdRp	RNA dependent RNA polymerase
RIG-I	retinoic acid inducible gene I
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
RVFV	Rift Valley fever virus
S	small genome segment
SDS-PAGE	sodiumdodecylsulphate-polyacrylamide gel electrophoresis
SK	Slovakia
Slo	Slovenia
SNV	Sin Nombre virus
STAT	signal transducer of activation and transcription
TBK-1	Traf family member-associated NFκB activator-binding kinase 1
TBST	Tris-buffered saline Tween 20
TLR	Toll-like receptor
TNF□	tumor necrosis factor
UV	ultraviolet
WB	Western blot
wt	wild-type

1. Introduction

1.1 Historical overview

The earliest description of a human viral disease possibly caused by hantaviruses dates back to the 10th century (Lee, 1982). In the 1930s Japanese physicians encountered this disease in Manchuria and also Russian scientists in Siberia, but the causative agent was not identified at that time. During the Korean War (1950-1953) about 3000 soldiers were hospitalised with an acute febrile illness. This disease was initially termed Korean hemorrhagic fever but is now referred to as hemorrhagic fever with renal syndrome (HFRS). It was not until 1978 that the virus was discovered; the first hantavirus described, named Hantaan virus after the nearby Hantaan river in Korea, was isolated from the striped field mouse, *Apodemus agrarius,* by Ho-Wang Lee and co-workers (Lee et al., 1978). Soon after that, other HFRS-associated hantaviruses were identified in Asia and Europe. Antibodies to hantaviruses were found in rodents and humans all over the world and new related viruses were isolated, e.g. Puumala virus (PUUV) from bank voles, *Clethrionomys glareolus*, in Finland*,* or Dobrava virus (DOBV) from the yellow-necked field mouse, *Apodemus flavicollis,* in Slovenia.

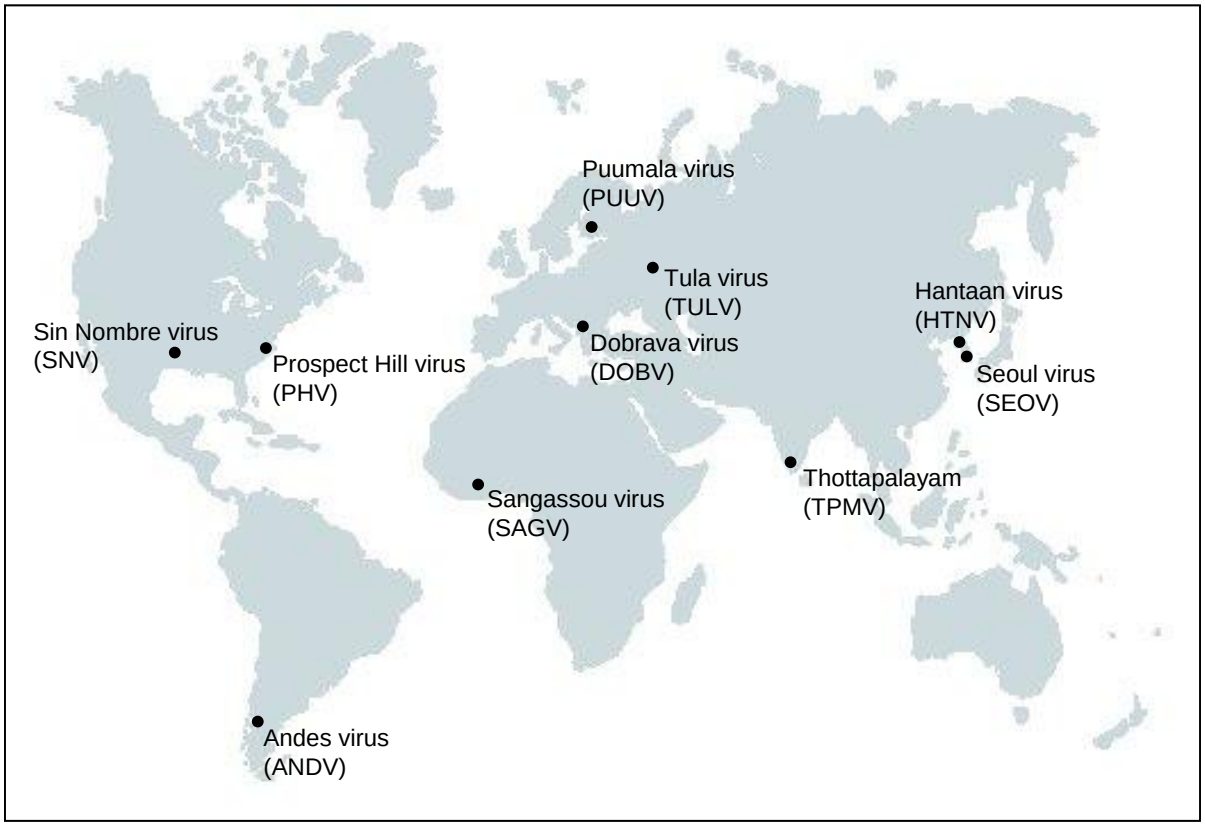

Figure 1: Global distribution of selected Hantaviruses. Indicated is the first isolation habitat.

The next important step in the history of hantavirus research was the discovery of a novel disease, hantavirus cardiopulmonary syndrome (HCPS), in the Americas. In May 1993 a major outbreak of an acute respiratory syndrome with a high fatality rate occurred in the Four Corners region of the southwestern part in the United States. Soon after, Sin Nombre virus (SNV) was discovered as a causative agent of this disease. This virus was shown to be carried by the common deer mouse, *Peromyscus maniculatus.* Later on it became clear that SNV and similar HCPS-associated hantaviruses, like Andes virus (ANDV), are present all over the Americas (Clement, 2003; Jones et al., 2008; Kruger et al., 2001; Peters and Khan, 2002; Figure 1). Interestingly, the Thottapalayam virus, isolated from the shrew *Suncus murinus*, an insectivore in India, was actually the first hantavirus isolated from a non-rodent reservoir (Carey et al., 1971). Recently, an increasing number of shrew-associated hantaviruses (as Tanganya virus) suggested a potential role of shrews as reservoirs for a novel group of hantaviruses. However, it is unclear whether these novel viruses can be transmitted to humans and cause disease (Arai et al., 2007; Arai et al., 2008; Klempa et al., 2007; Song et al., 2007).

1.2 Hantaviruses within the *Bunyaviridae* family

Hantaviruses form a separate genus within the *Bunyaviridae* family. The *Bunyaviridae* family contains five genera: Bunyavirus, Hantavirus, Nairovirus, Phlebovirus and Tospovirus (Table 1). It represents one of the largest viral families and comprises over 300 individual virus species. They are grouped in one family because of their common structure, including a trisegmented RNA genome of mostly negative polarity and a roughly similar protein coding pattern within each genome segment.

Table 1: Members of the family *Bunyaviridae.* Taxonomical classification according to the seventh report of The International Committee of Taxonomy of Viruses (ICTV) and selected significant pathogens (van Regenmortel et al., 2000)

Genus	Type species	Selected significant human/veterinary pathogens
Bunyavirus	Bunyamwera virus	La Crosse Virus Thyna Virus Akabane virus Oropouche virus
Hantavirus	Hantaan	Hantaan virus Sin Nombre virus
Nairovirus	Dugbe virus	Crimean-Congo hemorrhagic fever virus Nairobi sheep disease virus
Phlebovirus	Rift Valley fever virus	Rift Valley fever virus Sandfly fever-Sicilian virus
Tospovirus	Tomato spotted wilt virus	Tomato spotted wilt virus

Some of the viruses like Tahyna or LaCrosse viruses (genus Bunyavirus), Rift Valley fever virus (genus Phlebovirus), Hantaan virus (genus Hantavirus), and Crimean-Congo hemorrhagic fever virus (genus Nairovirus), cause important human diseases (Table 2). The bunyaviruses depend on wild animal hosts for their persistence in nature and because transmissions from human to human are not common, humans are considered to be dead-end hosts. With the exception of hantaviruses, Bunyaviruses are transmitted by arthropods to humans. The natural reservoirs of hantaviruses are rodents or insectivores of the family Muridae and Insectivores, respectively. The rodent-borne hantaviruses can be classified into four subfamilies: the Murinae- (family Muridae), Arvicolinae-, Neotominae- and Sigmondontinae- (family Cricetidae) associated viruses. Recently several insectivore-borne hantaviruses were isolated from shrews belonging to the family Soricidae (Maes et al., 2009). It could be shown by nucleotide sequence analyses that hantaviruses cluster into genetic lineages which are associated with the genetic lineages of their natural reservoirs´ hosts (Kruger et al., 2001). Currently 23

hantavirus species are listed in the latest International Committee of Taxonomy of Viruses report (Fauquet et al., 2005).

Table 2: Selected hantavirus species and their rodent hosts, geographical distribution, and human disease caused by them (Schonrich et al., 2008)

Species	**Abbreviation**	**Rodent host**	**Distribution**	**Disease**	**Case fatality**
Murinae-associated					
Dobrava Belgrade virus	DOBV (Aa)	*Apodemus agrarius*	Central and East Europe	HFRS	0.9%
	DOBV (Af)	*Apodemus flavicollis*	South-East Europe	HFRS	9-12%
	DOBV (Ap)	*Apodemus ponticus*	South of European Russia	HFRS	6.5%
Hantaan virus	HTNV	*Apodemus agrarius*	Asia	HFRS	≤ 15%
Sangassou virus	SANGV	*Hylomyscus alleni (simus)*	Africa (Guinea)	unknown	unknown
Seoul virus	SEOV	*Rattus norvegicus*	Asia	HFRS	1-2%
Arvicolinae-associated					
Prospect Hill virus	PHV	*Microtus pennsylvanicus*	North America	unknown	unknown
Puumala virus	PUUV	*Clethrionomys glareolus* *Clethrionomys rufocanus*	Eastern and Southern Europe	HFRS (mild)	< 1%
Tula virus	TULV	*Microtus arvalis* *Microtus rossiaemeridionalis*	Central and East Europe	unknown	unknown
Sigmodontinae-associated					
Andes virus	ANDV	*Oligoryzomys longicaudatus*	Argentina	HCPS	43-56%
Black Creek Canal virus	BCCV	*Sigmodon hispidus*	North America	HCPS	
Neotominae-associated					
Sin Nombre virus	SNV	*Peromyscus maniculatus*	North America	HCPS	35%
Insectivore-associated					
Thottapalayam virus	TPMV	*Suncus murinus*	India	unknown	unknown
Tanganya virus	TGNV	*Crocidura theresea*	Guinea	unknown	unknown
Seewis virus	SWSV	*Sorex araneus*	Switzerland	unknown	unknown
Camp Ripley virus	RPLV	*Blarina brevicauda*	USA	unknown	unknown

1.3 Genome structure and replication

The virus genome consists of three single-stranded RNA segments of negative polarity: the large (L) segment encoding the viral RNA-depended RNA polymerase (RdRp), the medium (M) segment encoding the glycoprotein precursor of two envelope glycoproteins (G1 or G_N and G2 or G_C), and the small (S) segment encoding the nucleocapsid (N) protein. Virions are of spherical or ovoid morphology and the lipid bi-layered envelope has a diameter of 80 – 120 nm (Figure 2).

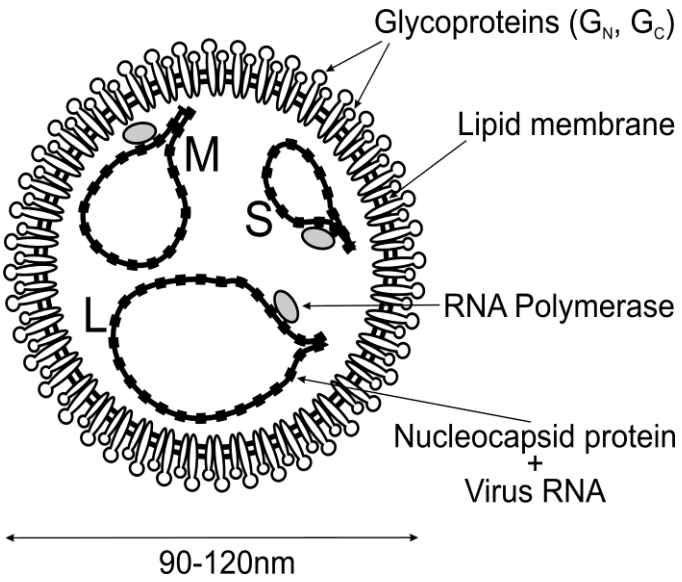

Figure 2: Scheme of a hantavirus particle.

The three RNA segments are associated with the N protein to form ribonucleocapsid structures. The RdRp acts as a replicase, transcriptase, endonuclease and possibly RNA helicase. The glycoprotein precursor is co-translationally cleaved into G1 and G2 proteins, which are thought to form a heterodimer. An open reading frame (ORF) for a putative non-structural protein (NS) has been found in all hantaviruses associated with *Arvicolinae* (e.g. PUUV, PHV, TULV) or *Neotominae*-, and *Sigmodontinae* rodents (e.g. SNV, ANDV respectively) but not in *Murinae*-associated hantaviruses (HTNV, SEOV, DOBV).

The 5´and the 3´-termini of all three genome segments are genus specific, highly conserved and complementary to each other. This makes it possible to form panhandle structures of the RNA segments, which are typical for bunyaviruses. These structures are thought to play a role in viral transcription and in the proposed prime-and-realign mechanism of replication. Detailed analyses of the molecular mechanisms involved in

the initiation of transcription and replication is hampered, as no suitable reverse genetics system for production of recombinant infectious virions has been established.
After binding to the cellular receptor the virus was enabled to enter the cell by clathrin-dependent endocytosis, and upon internalisation, the three ribonucleocapsid complexes are released into the cytoplasm together with the virion-associated RdRp. Shortly after entry and uncoating of the virion in the cytoplasm, full-length complementary strands of each segment are synthesised, serving as template for vRNA synthesis (Garcin et al., 1995; Plyusnin et al., 1996; Schmaljohn and Nichol, 2001).
At first the N proteins as well as the RdRp are translated in the cytoplasm at the ribosomes. After synthesis of the glycoprotein precursor at the endoplasmic reticulum, the precursor is cleaved into the two glycoproteins G1 and G2. These are in turn transported to the Golgi apparatus for glycosylation and accumulate as heterodimers (Alfadhli et al., 2001; Dunn et al., 1995; Shi and Elliott, 2002) ready for assembly of new virions. At the step where the assembly of new virions is started, the possibility of genetic reassortment is given (Schmaljohn and Nichol, 2001).

1.4 Pathogenesis

1.4.1 Infection in natural hosts and humans

In humans, hantaviruses cause HFRS or HCPS depending on the viral strain involved. Asymptomatically infected rodents are the virus reservoir. Transmission to humans takes place through aerosols containing hosts´ excretions. In rodents and humans, primary replication occurs mainly in pulmonary endothelial cells and macrophages. Although neutralising antibodies appear in rodents soon after infection, hantaviruses persist in the host. Humans also develop neutralising antibodies but are able to clear the virus. This suggests that hantaviruses regulate cellular host response in rodents to effect viral persistence (Mackow and Gavrilovskaya, 2001). Recently it was shown that HTNV can productively infect dendritic cells, upregulating the costimulatory major histocompatibility complex (MHC) and adhesion molecules and inducing release of proinflammatory cytokines. This supports the hypothesis that in humans, hantavirus elicits a strong immune response which could be an essential part of the virus-associated pathogenesis (Raftery et al., 2002).

1.4.2 Hemorrhagic fever with renal syndrome (HFRS)

The clinical course of HFRS can be divided into five distinct phases: febrile phase, hypotensive phase, oliguric phase, diuretic phase, convalescent phase. After an incubation period of two to four weeks the febrile phase (three to seven days) starts with an abrupt onset of flu-like symptoms such as chills, general malaise, headache, nausea, back and abdominal pain. At the end of this phase, conjunctival hemorrhages and petechiae occur. The hypotensive phase is characterised by a drop of blood platelets and can last from several hours up to two days. Severe cases are associated with irreversible shock and one-third of lethal HFRS cases occur during this stage. The oliguric phase (three to seven days) is characterised by massive proteinuria which occurs due to renal failure. Typical findings are elevated concentrations of serum creatinine and urea. Blood pressure becomes normalised or changes to hypertension. The progression to the diuretic phase is a positive prognostic sign for the patient. Diuresis of three to six liters is usually observed. The convalescent phase is characterised by normalisation of the clinical and biochemical markers (Kruger et al., 2001).

HFRS is common in Europe and Asia. The most severe forms of HFRS are caused by HTNV in eastern Russia, China and Korea. In Europe, severe cases occur mostly in the Balkan region and are caused by DOBV. A milder form of HFRS in Europe, called Nephropathia epidemica, is caused by Puumala virus.

1.4.3 Hantavirus Cardio Pulmonary Syndrome (HCPS)

Clinical features of HCPS can be divided into four phases (febrile, cardiopulmonary, diuretic, and convalescent). The febrile phase typically lasts three to five days and is characterised by fever, myalgia and malaise. Other symptoms such as headache, dizziness, anorexia, nausea, vomiting, diarrhea and abdominal pain may occur.

Early diagnosis at this stage is difficult as prodromal symptoms are unspecific. This phase can progress with pulmonary edema, non-productive cough and tachypnea. The cardiopulmonary phase is characterised by the presentation of shock and pulmonary edema. Shock can be accompanied by oliguria. Once pulmonary edema is present, the disease proceeds fast. Patients can die within 24-48h; hypoxia, circulatory compromise, or both being the immediate cause of death. In the diuretic phase, rapid clearances of pulmonary edema as well as resolution of fever and shock occur. Spontaneous diuresis is an early sign of this process. Convalescent phase may last up to two months, with

patients recovering apparently completely. However, continued follow-up is necessary to determine the long-term persistence of pulmonary dysfunction.
HCPS is common in the Americas and caused by Sin Nombre virus, New York virus, Bayo virus, Black Creek Canal virus and Andes virus (Kruger et al., 2001).

1.5 Innate immunity

1.5.1 Hantaviruses and the immune system

The host immune response against pathogens can be broadly divided into an adaptive and an innate part. The adaptive immune response consists of specific pathogen recognition by cellular (T cells) and humoral (B cells) mechanisms. In this study we focus on the innate immune response which provides the first line of defence against pathogens. Innate immune responses can be elicited by cellular pathogen recognition receptors (PRR), which recognise characteristic pathogen-associated molecular patterns (PAMPs) such as genomic DNA and RNA or double-stranded RNA (dsRNA) (Kawai and Akira, 2006).
The best characterised PRRs are the retinoic acid-inducible gene I (RIG-I), and the toll-like receptor 3 (TLR3). Recruitment of these PRRs leads to the production of type I interferons (IFNs) (Takeuchi and Akira, 2009). Some viral infections produce dsRNA. RIG-I and also TLR3 directs IFN signalling responses through binding of dsRNA, which leads to the induction of a signal cascade that leads to the activation of the interferon regulatory factor 3 (IRF3). IRF3 is phosphorylated and then forms a homodimer, which translocates into the nucleus. Coordinated binding of IRF3 and other transcription factors to the IFN-β promoter leads to induction of IFN-β (Akira and Takeda, 2004). The secreted IFN-β binds to IFN-α/β receptors (IFNARs) which activate JAK/STAT signalling pathways and direct transcription of a huge set of different interferon-stimulated genes (ISGs), which constitute an antiviral state.
This antiviral state can efficiently block replication of hantaviruses. Interestingly, pathogenic and non-pathogenic hantaviruses differentially induce innate immune responses. Pathogenic hantaviruses are proposed to counteract innate immunity by blocking signalling pathways which lead to IFN expression. In recent studies, it was discovered that the G1 cytoplasmic tail of the pathogenic New York virus (NYV), but not the G1 derived from the non-pathogenic Prospect Hill virus (PHV), blocks RIG-I signalling upstream of the IRF3, resulting in the inhibition of the transcription from IFN-β promoters and ISREs (Alff et al., 2006; Spiropoulou et al., 2007). Furthermore, it was

shown that TLR3 is involved in recognition of the pathogenic HTNV but not required for the induction of innate responses by PHV (Handke et al., 2009).

1.5.3 Interferon system

The IFN molecules represent a group of secreted cytokines that elicit distinct antiviral effects. According to their amino acid sequence, they are grouped in three classes: Type I, II and type III IFN. Type I IFN comprises IFN-α, -β, -ω, -ε, -δ, -τ, and –K. Type II IFN is represented by IFN-γ and Type III interferon induces IFN-λ1, - λ2, and -λ3 (Randall and Goodbourn, 2008).

Type I IFNs are of crucial importance for the induction of a variety of innate antiviral response mechanisms. The antiviral effect of IFNs is mediated by IFN-induced proteins which inhibit the propagation of viruses by distinct mechanisms.

IFN-induced Mx proteins are large GTPases related to dynamin and the intrinsic GTPase activity of the MxA protein is required for its antiviral activity (Pitossi et al., 1993). It was shown that Mx proteins are able to block negative-stranded RNA viruses (e.g. Influenza A and C) (Frese et al., 1996; Staeheli et al., 1993). The human MxA protein is known to have antiviral properties against different genera of the family *Bunyaviridae*, including Hantavirus, Orthobunyavirus (LACV), Nairovirus (Dugbe virus, CCHFV), and Phlebovirus (RVFV) (Andersson et al., 2004; Bridgen et al., 2004; Frese et al., 1996). In cells stimulated with type I IFN, MxA accumulates and can block replication by direct interaction with viral proteins (Staeheli et al., 1993). *In vitro* studies with transfected Vero E6 cells which constitutively express MxA showed that the replication of HTNV was inhibited (Frese et al., 1996).

1.6 Dobrava-Belgrade hantavirus

Dobrava-Belgrade virus (DOBV) is intensively studied because of its unique properties; different virus lineages exist in different regions of Europe. These lineages are harboured by different host reservoirs and probably display different levels of virulence in humans. DOBV seems to be the most pathogenic European hantavirus. The severity of DOBV-associated HFRS can reach a death rate of up to 12% as reported from the Balkans (Antoniadis et al., 1996; Avsic-Zupanc et al., 1992; Lundkvist et al., 1997; Papa et al., 1998). Interestingly, DOBV is carried by three distinct reservoir rodent species *Apodemus flavicollis* (yellow-necked field mouse), *Apodemus agrarius* (striped field mouse) and *Apodemus ponticus* (Caucasian wood mouse) (Figure 3).

The DOBV prototype strain (named Dobrava-Slovenia or Slo/Af for short) was isolated from lungs of *A. flavicollis* captured near the Dobrava village in Slovenia (south-east Europe) (Avsic-Zupanc et al., 1992; Avsic-Zupanc et al., 1995). Simultaneously the same virus was found also in a HFRS patient from Belgrade (Gligic et al., 1992). The International Committee on Taxonomy of Viruses (ICTV) proposed the name Dobrava-Belgrade virus (DOBV) for this virus species (Fauquet et al., 2005).

In contrast, in eastern, north-eastern, and central Europe, DOBV is carried by *A. agrarius*. Dobrava-Saaremaa was isolated in Estonia, north-eastern Europe, and was suggested to represent the prototype for a unique virus species (Plyusnin, 2002). Subsequently, another DOBV representative (named Slovakia or SK/Aa for short) was isolated from *A. agrarius* trapped in Slovakia, central Europe.

Recently, a fourth DOBV was detected in still another rodent host, *A. ponticus*, captured in Sochi district, in the southern part of European Russia. Detailed sequence and phylogenetic analysis showed that the strains from *A. ponticus* form a distinct lineage, DOBV-Ap (Klempa et al., 2008).

Detailed phylogenetic analyses of these newly isolated viruses showed that these three DOBV strains form three genetic lineages, DOBV-Af, DOBV-Aa, and DOBV-Ap with regard to their reservoirs hosts *A. flavicollis*, *A. agrarius*, and *A. ponticus,* respectively.

The most severe HFRS cases have been reported from the Balkans. The infectious virus carrier in this region was identified as *A. flavicollis* (Avsic-Zupanc et al., 1992). In central Europe HFRS infections are caused by viruses of the DOBV-Aa lineage. The severity of HFRS caused by DOBV-Aa is mild to moderate, but usually less severe than clinical courses in the Balkans, where the disease is associated with DOBV-Af lineage.

Figure 3: Natural hosts of DOBV hantavirus. Striped field mouse *Apodemus agrarius,* carrier of DOBV-Aa (left), Yellow-necked field mouse, *Apodemus flavicollis,* carrier of DOBV-Af (middle) (picture by Dr. B. Klempa), and *Apodemus ponticus* carrier of DOBV-Ap (right) (picture by E. Tkachenko)

1.7 Genetic reassortment

Genetic reassortment is the process of intracellular exchange of genetic material between two or more viruses. For RNA viruses with segmented genomes, this phenomenon is described for several species. Reassortment plays an important role in the evolution of these viruses and can significantly change such properties as antigenicity, host range, epidemiology, and virulence of the viruses. Influenza viruses are the best known and described examples for the exchange of genome segments (Zhou et al., 1999). Within the *Bunyaviridae* family, reassortment has been described for members of the genera Phlebovirus (Rift Valley Fever Virus), Orthobunyavirus (La Crosse, Snoswshoe hare virus), Tospovirus (Tomato spotted wilt virus), Nairovirus (Crimean-Congo hemorrhagic fever virus) (Hewson et al., 2004; Qiu et al., 1998; Sall et al., 1999; Urquidi and Bishop, 1992). Genetic reassortment in nature was also described for hantaviruses. Natural reassortment can occur between related viruses which circulate in the same habitat and are able to infect the same host animal. The possibility of natural genetic reassortment was described for HCPS-associated hantaviruses. Complete nucleotide sequence analysis of the S and M segment, of Sin Nombre virus isolates revealed that genetically distinct viruses co-circulate in nature and that these viruses can undergo gene segment reassortment (Henderson et al., 1995; Li et al., 1995). Recently, genetic reassortment in nature was also described for HFRS-associated hantaviruses (Zou et al., 2008).

To determine the ability of genetic reassortment between distinct and closely related hantaviruses, in vitro studies of HCPS-associated hantaviruses were undertaken (McElroy et al., 2004; Rizvanov et al., 2004; Rodriguez et al., 1998).

Rodriguez et al. (1998) co-infected VeroE6 cells with two closely related Sin Nombre virus strains and, furthermore, they performed co-infections with Sin Nombre and Black Creek Canal virus, which are more distantly related. In this study, genetic reassortment was frequently detected between closely related SNV strains, but rarely found between SNV and BCCV. In later studies, McElroy et al. (2004) and also Rizvanov et al. (2004) examined genetic reassortment between ANDV and SNV. Both groups found reassortants between these two distinct hantaviruses. Using these reassorted viruses it was found that the M-Segment did not contain the decisive determinant for lethality caused in Syrian hamsters by the Andes virus (McElroy et al., 2004).

It has been suggested that natural genome reassortment occurs also in the case of DOBV. DOBV-Saaremaa, isolated from *A. agrarius*, in Estonia, was suggested to be involved in reassortment processes between the DOBV-Aa and DOBV-Af lineages (Klempa et al., 2003).

Although a large overlap between the distribution of *A. agrarius* and *A. flavicollis* exists in Europe, so far the two host-specific subtypes have been detected sympatrically only in the border region between central and south-eastern Europe (Avsic-Zupanc et al., 2000; Sibold et al., 2001). The coexistence of two different virus lineages in the same geographic region indicates some genetic stability and evolutionary distinctness (Henderson et al., 1995; Klempa et al., 2003; Razzauti et al., 2008; Zou et al., 2008).

So far, no *in vitro* reassortment studies between European hantaviruses have been performed

1.7.1 Reassortment scenarios

Since the genome of hantaviruses consists of three genome segments there are six possibilities for reassortment processes between two viruses (Figure 4). Theoretically, each genome segment can be exchanged after co-infection with two viral strains.

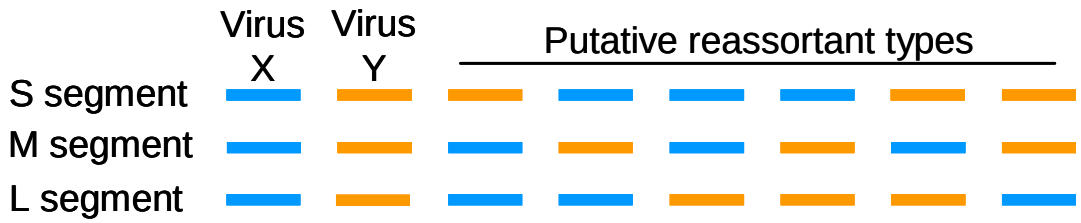

Figure 4: Schematic diagram of all possible reassortment scenarios between two viruses with three genome segments. Theoretically every genome segment can be exchanged.

Furthermore both versions of the particular genome segment can be found within certain virions, as described in earlier studies with HCPS-associated hantaviruses in cell culture (Rizvanov et al., 2004; Rodriguez et al., 1998).

1.8 Aim of this study

The pathogenesis of hantavirus diseases and, in particular, the genetic basis of the different virulence of the various hantaviruses towards humans is not well understood. For hantaviruses, there is no reverse genetics system which could help to assign particular virulence immune modulation properties to particular segments or even nucleotide positions of the viral genome.

Therefore, the aim of this work was to establish reassortants between the two DOBV lineages SK/Aa and Slo/Af *in vitro*. The DOBV-Aa x DOBV-Af reassortants are used to find markers of immunomodulation which are distinct for DOBV-Aa and DOBV-Af, and to assign these markers to particular genomic segments.

2. Material

2.1 Virus strains

Table 3: List of virus strains

Virus strain	Source
SK/Aa	Dr. Klempa, Berlin
Slo/Af	Dr. Avsic-Zupanc, Ljubljana
PHV (type 3571)	Dr. Robert Tesh, Galveston
HTNV (strain 76-118)	Dr. Åke Lundkvist, Stockholm

Table 4: List of reassorted viral clones with genotype afa
(S and L segment of SK/Aa origin and M segment of Slo/Af origin)

Code of virus clone	Source
A6	Kirsanovs et al., this study
B13	Kirsanovs et al., this study

Table 5: List of reassorted viral clones with genotype faf
(S and L segment of Slo/Af origin and M segment of SK/Aa origin)

Code of virus clone	Source
A36	Kirsanovs et al., this study
B5	Kirsanovs et al., this study
A35	Kirsanovs et al., this study

2.2 Cell lines

Table 6: List of cell lines used in this work

Cell line	Origin	Reference
VeroE6	green monkey epithelial kidney cells	ATCC Nr.: CRL-1586™
A549	human epithelial lung cells	ATCC nr.: CCL-185™
A549 ΔRIG-I knockdown	human epithelial lung cells	kindly provided by Markus Matthäi, Robert Koch-Institute, Berlin, Germany
HuH7	Human hepatoma cells	(Nakabayashi et al., 1982)

2.3 Reagents

1 kb DNA Ladder	Fermentas (St. Leon-Rot, Germany)
100 bp DNA Ladder	Fermentas (St. Leon-Rot, Germany)
6x Loading Buffer	Fermentas (St. Leon-Rot, Germany)
Agarose	Biozym (Hess. Oldendorf, Germany)
APS (Ammoniumpersulphate)	Roth (Karlsruhe, Germany)
Avicel	FMC Biopolymer (Philadelphia, USA)
Betaine Enhancer	Biomol (Hamburg, Germany)
BME 10x	Biochrom AG (Berlin, Germany)
Boric acid	Merck (Darmstadt, Germany)
D-MEM	Biochrom AG (Berlin, Germany)
dNTP (25 mM each)	Bioline (Luckenwalde, Germany)
DTT (0.1 M)	Invitrogen (Karlsruhe, Germany)
EDTA	Invitrogen (Karlsruhe, Germany)
E-MEM	Biochrom AG (Berlin, Germany)
Ethanol	JT Baker (Griesheim, Germany)
Ethidiumbromide	Roth (Karlsruhe, Germany)
FCS	Perbio (Bonn, Germany)
Gentamycinsulphate	Bio - Wittacker
HBSS	Gibco/Invitrogen (Karlsruhe, Germany)
Hepes	Biochrom AG (Berlin, Germany)
Hotstart PCR Mix	Biomol (Hamburg, Germany)
L-Alanyl-Glutamine	Biochrom AG (Berlin, Germany)
Lipofectamin 2000	Invitrogen (Karlsruhe, Germany)
Methanol	JT Baker (Griesheim, Germany)
$MgCl_2$ (25 mM)	Perkin Elmer (Waltham Massachusettes, USA)
M-MLV-Reverse Transcriptase (600 U/µl)	Invitrogen (Karlsruhe, Germany)
N6-Primer (600 ng/µl)	Fa. Pharmacia Biotech (Dübendorf, Germany)
Non essential amino acids	Biochrom AG (Berlin, Germany)
OptiMEM	Gibco/Invitrogen (Karlsruhe, Germany)
PBS	Sigma-Aldrich, (Deisendorf, Germany)
PCR Buffer 10x	Perkin Elmer (Waltham Massachusettes, USA)
PCR Mix	Biomol
Penicillin/Streptomycin (10000 U/ml)	Biochrom (Hamburg, Germany)
RNA Safe	Fermemtas (St. Leon-Rot, Germany)
Rotiphorese-Acrylamide	Roth(Karlsruhe, Germany)
Sodiumbicarbonate	Biochrom AG (Berlin, Germany)
Sodiumpyruvate	Biochrom AG (Berlin, Germany)
Tris base	Roth (Karlsruhe, Germany)
Trypsin	Invitrogen (Karlsruhe, Germany)
Tween 20	Sigma-Aldrich (Deisendorf, Germany)
Ultra pure water	PAA (Pasching, Austria)

2.4 Consumables

Cell culture flasks (T25, T75, T125)	Sarstedt (Nürmbrecht, Germany)
Cell culture dishes (10 cm)	Sarstedt (Nürmbrecht, Germany)
Falcon tubes (15 ml, 50 ml)	Sarstedt (Nürmbrecht, Germany)
Petri dishes	Sarstedt (Nürmbrecht, Germany)
Serological pipettes (5 ml, 10 ml, 25 ml)	Sarstedt (Nürmbrecht, Germany)
Nitrocellulose Membrane Protran	Whatman (Dassel, Germany)
Nitrocellulose Membrane Protran	Schleicher und Schuell (Dassel, Germany)
6-, 12-, 24-well Plates	Nunc (Wiesbaden, Germany)

2.5 Buffers

Titration antibody dilution buffer	PBS 0.1% Tween 20 5 % FCS
Titration washing buffer	PBS 0.15 % Tween 20
Virus dilution buffer	25 ml HBSS 500 µl Hepes 500 µl Streptomycin/Penicillin 250 µl FCS
Titration Overlay	1:1 mixture of 2.4% Avicel and basal Eagle´s medium (2x BME) supplemented with 5% FCS, 2.5% Hepes and antibiotics
Agarose overlay	1:1 mixture of 2.4% Agarose and basal Eagle´s medium (2x BME) supplemented with 5% FCS, 2.5% Hepes and antibiotics
10x TBE	108 g Tris base 55 g boric acid 3.72 g EDTA fill up to 1000 ml with double distilled water

2 x SDS sample buffer	1.2 ml	H_2O
	8.3 ml	0.5 M Tris-HCl (pH 6.8)
	6 ml	10 % SDS (w/v)
	1.5 ml	glycerine
	9 mg/ml	bromphenol blue
	5 %	ß-mercaptoethanol
10 x SDS electrophoresis buffer	Tris	250 mM
	Glycine	1.92 M
	SDS	10 g/l
Semidry Blotting buffer	48 mM Tris	
	39 mM glycine	
	1.3 mM SDS	
	20 % methanol	
TBST	100 mM Tris-HCl (pH 8.0)	
	1.5 M NaCl	
	0.5 % Tween 20	
Ponceau Red	0.1 % Ponceau red	
	1 % acetic acid	

2.6 Solutions

1x PBS

9.55 g PBS
fill up to 1000 ml with double distilled water

Avicel (2.4%)

24 g Avicel
fill up to 1000 ml with double distilled water

2x BME

350 ml ultra pure H_2O
40 ml $NaHCO_3$
10 ml stable L-glutamine
100 ml 10x BME

Cell culture media

Minimal essential medium (MEM) with Earle´s Salt, 25 mM Hepes
To 500 ml MEM were added:

- 50 ml FCS
- 5 ml glutamine
- 5 ml natriumpyruvat
- 5 ml non essential amino acids
- 500 µl gentamycinsulphate

2.7 Kits

A-addition Kit	Qiagen (Hilden, Germany)
Autoread Kit	Amersham Pharmacia Biotech (Piscataway, USA)
Big dye Terminator V1.1 Cycle Seq Kit	Applied Biosystems (Darmstadt, Germany)
DNA Terminator End repair Kit	Lucigen (Middleton, WI USA)
LightCycler®Faststart DNA Master^{Plus}HybProbe	Roche (Mannheim, Germany)
Luciferase Reporter Gene Assay, constant light signal	Roche (Mannheim, Germany)
QIAGEN Plasmid Mini Kit	Qiagen (Hilden, Germany)
QIAmp Viral RNA Mini Kit	Qiagen (Hilden, Germany)
StrataClone^{TM}PCR Cloning Kit	Stratagene (LaJolla, Canada)
Super Signal West Dura Extended duration substrate	Pierce (Rockford, USA)
Topo TA-Cloning Kit	Invitrogen (Karlsruhe, Germany)
Venor®*GeM* Mycoplasma Detection Kit	Minerva Biolabs (Berlin, Germany)

2.8 Antibodies

Table 7: Primary antibodies for hantavirus focus assay and western blot analysis

Detection specificity	**Source**	**Reference**	**Dilution**
α – SK/Aa	rabbit	Razanskiene et al., 2004	1:500
α – Slo/Af	rabbit	Razanskiene et al., 2004	1:500
α – PHV	rabbit	Sibold et al., 1999b	1:1000
M 143 α – MxA	mouse	Flohr et al., 1999	1:1000
α – RIG-I	rabbit	Axxora, Lörrach, Germany	1:1000
ab6276 α -Actin (beta)	mouse	Abcam plc, Cambridge, UK	1:150000

Table 8: Secondary antibodies for hantavirus focus assay and western blot analysis

Antibody	**Dilution**	**Manufacturer**
horseradish-peroxidase (HRP)-labelled anti-human immune globulin	1:1000	Dianova (Hamburg, Germany)
horseradish-peroxidase (HRP)-labelled Goat anti-Rabbit IgG	1:1000	Pierce (Rockford, USA)
horseradish-peroxidase (HRP)-labelled Goat anti-Mouse IgG	1:1000	Pierce (Rockford, USA)

2.9 Primers

Table 9: List of primers used for Multiplex PCR

Primer name[a]	Primer sequence 5´ → 3´	Fragment size in basepairs
SKS-F	TGG GTC CAT ATC ATC GC	
SKS-R	AGG GTT GAA GAG TGG TTA GAT	510
SKM F	CGC TGG CAC ACA AAG TTA ATC	
SKM-R	GCT GGA AAT CAA GAC GGA ACT G	293
SKL-F	AAT CCC CTT ATC AAC GTC AG	
SKL-R	TTG CTG GAG TAC CGA ATA ATG	176
SloS-F	TCA GGA TCC ATA TCG TCA C	
SloS-R	CGG GTT GAA TGG CTT GAC C	510
SloM-F	GGA AGT CAA GAT GGG CCT ATT	
SloM-R	CGC TGG CAT ACG AAA TTG ACT	293
SloL-F	CCT GCC TCA TAC TTA AGC CTT	
SloL-R	TTG CTG GGG TGC CTA ATA ATG	205

[a]Primer name composed of the virus strains (SK = DOBV/Aa, Slo = DOBV/Af), segment (S, M, or L) and sense (F = forward primer, R = reverse)

Table 10: List of primers used for PCR and sequencing. Indicated are the name of the primer, target virus, the segment and the orientation of the primer.

Primer	Target	Seg.	forward reverse	Nucleotide position	Primer sequence 5´→ 3´
RT DOB	SK/Aa	S	f/r	1-22	TAG TAG TAK RCT CCC TAA ARA G
D955C	SK/Aa	S		955-935	ACC CAI ATT GAT GA(I+C) GGT GA
D357F	SK/Aa	S	F	357-376	GA(I+C) ATT GAT GAA CCI ACA GG
D1162C	SK/Aa	S		1162-1142	AGT TGI AT(I+C) CCC ATI GA(I+C) TGT
D113	A35, B13, B5	S		114-137	GAT GCA GAI AAI CAI TAT GAR AA
M11	Slo/Af, SK/Aa	M	F	11-29	CTC CGC AAG AAA TAG CAG T
M3618	Slo/Af, SK/Aa	M	R	3638-3618	GCA AGA TAT AGA AAT ACC CAC
G1NS	Slo/Af, SK/Aa	M			aaa tct aga cac ATG ATC ATG TGG GGT CTA CTA TTG ACA
MD3643	Slo/Af, SK/Aa	M	R	3635-3611	CTC CGC AAG ATA TAG AAA TAC CCA C
MD	A6, A36	M	F	1-20	TAG TAG TAG RCT CCG CAA GA
M2029R	A6, A36	M	R	2029-2009	CCA TGI GCI TTI TCI KTC CA
M1990R	A6, A36	M	R	1990-1970	TCI GMT GCI STI GCI GCC CA
M1470	A6, A36	M		1469-1488	CCI GGI TTI CAT GGI TGG GC
M1674	A6, A36	M		1674-1698	TGT GAI RTI TGI AAI TAI GAG TGT GA
M1674	A6, A36	M		1674-1698	TGT GAI RTI TGI AAI TAI GAG TGT GA
M905F	A6, A36	M	F	905F	GTT GCA ACT TAT TCA ATT G
DOBM	A6, A36	M	R	1002R	CTA GGR ATI CCT GAR AAT GCA G
M11	B5	M		11-29	CTC CGC AAG AAA TAG CAG T
M1851	B5	M		1851-1873	CAA TAA CAC CTC AGT CAA CAA GTC
SK-L1-F	SK/Aa, A6, B13	L	F	1	GAG AAATAT AGA GAA ATT CAT AG
SK-L-R1	SK/Aa, A6, B13	L	R	1	AAC CAG TCA GTT CCA TCA TC
SK-L-F1	SK/Aa, A6, B13	L	F	1	ATG TAT GTG AGT GCA GAT GCA A

Primer	Target	Seg.	forward reverse	Nucleotide position	Primer sequence 5´→ 3´
SK-L3-R	SK/Aa, A6, B13	L	R	3	GTA GAA AGT TGA AAC AGA ATC C
Slo-L1-F	Slo/Af, A35, A36, B5	L	F	1	GAG AAA TAC AGA GAA ATT CAC AG
Slo-L-R1	Slo/Af, A35, A36, B5	L	R	1	AAC CAA TCA GTT CCA TCA TC
Slo-L-F1	Slo/Af, A35, A36, B5	L	F	1	ATG TAT GTA AGC GCT GAT GCA A
Slo-L3-R	Slo/Af, A35, A36, B5	L	R	3	GTA GAA AGT TGA GAC AGA ATC C
M13Uni	Plasmid				AGG GTT TTC CCAGTC ACG ACG TT
M13Rev	Plasmid				GAG CGG ATA ACA ATT TCA CAC AGG

2.10 Equipment

Centrifuges
Centrifuge 5417R — Eppendorf (Hamburg, Germany)
Centrifuge 5415 C — Eppendorf (Hamburg, Germany)
Centrifuge Biofuge pico — Heraeus (Kleinostheim, Germany)
Centrifuge Megafuge 1.0 — Heraeus (Kleinostheim, Germany)

Counting chamber
Neubauer improved — Marienfeld (Lauda-Königshofen, Germany)

Detection systems
CCD-Kamera — Bioblock Scientific (Illkirch, France)
DIANA II-CCD — Raytest (Straubenhardt, Germany)

Electrophoresis and blot systems
Elektrophorese Kammer — VWR International (Wien, Austria)
Elektrophorese-Kammer Mini — Bio-Rad (München, Germany)
Mini-Protean III — Bio Rad (München, Germany)
Semi-Dry Blot-Kammer — Owi (Porthmouth, Netherlands)

Incubators
Cellstar — Heraeus (Kleinostheim, Germany)
Hera Cell 150 — Heraeus (Kleinostheim, Germany)
CO_2 –Inkubator — Heraeus (Kleinostheim, Germany)

Laminar flows
Laminarbox Herasafe — Heraeus (Kleinostheim, Germany)

Luminometer
Berthold Mithras LB940 — Berthold (Bad Wildbach, Germany)

Microscopes
Lichtmikroskop Axiovert 40C — Zeiss (Oberkochen, Germany)

Mixer
Vortexer VV3 — VWR International (Wien, Austria)

PCR cyclers	
Master Cycler personal	Eppendorf (Hamburg, Germany)
pH meter	
pH Meter 766 Calimatic	Knick (Berlin, Germany)
Photometer	
Spektrophotometer Ultraspec 4000	Amersham Pharmacia Biotech (Piscataway, USA)
Power supplies	
Powersupply EPS 301	Amersham Pharmacia Biotech (Piscataway, USA)
Powersupply LKP 500/400	Amersham Pharmacia Biotech (Piscataway, USA)
Refrigerator combinations	
Kombi-Kühlschrank Glassline	Liebherr (Ochsenhausen, Germany)
Liebherr Premium	Liebherr (Ochsenhausen, Germany)
Liebherr Comfort NoFrost	Liebherr (Ochsenhausen, Germany)
Fridge Gram low energy	Gram (Sarstedt, Germany)
Scales	
Feinwaage	Sartorius (Göttingen, Germany)
Universalwaage	Sartorius (Göttingen, Germany)
Sequencer	
3130 Genetic Anlyzer	Applied Biosystems (Darmstadt, Germany)
Steam autoclave	
Durchreicheautoklav	Getinge (Rastatt, Germany)
Thermostats	
blockheater H250	Roth (Karlsruhe, Germany)
Blockheater Dri-Block DB3A	Techne (Burlington, USA)
Transilluminator	
BioDoc Analyze	Biometra (Göttingen, Germany)
Water bath	
Wasserbad WTE var 3185	Assistant (Sondheim, Germany)
Software	
TINA	Raytest (Straubenhardt, Germany)

3. Methods

3.1 RNA extraction

3.1.1 Extraction of RNA from cell culture supernatant

For isolation of viral RNA from cell culture supernatant, the QIAmp Viral RNA Mini Kit (Qiagen, Hilden, Germany) was used. The RNA was extracted according to the standard QIAmp viral RNA mini spin protocol. Briefly, 140 µl of cell culture supernatant was added to 560 µl AVL buffer containing carrier RNA and incubated at room temperature for ten minutes. Ethanol was added to the lysate and the lysate was applied to the column provided and centrifuged. RNA was then washed with washing buffers AW1 and AW2. An additional centrifugation step was performed to remove possible residues of the buffers. 60 µl buffer AVE were then added into the column, incubated for one min and the RNA was eluted by centrifugation. The RNA was stored at -20°C until further use.

3.1.2 Extraction of RNA from cell culture

The RNeasy Mini Kit (Qiagen, Hilden, Germany) was used for isolating viral RNA from cells. The RNA was extracted according to the standard protocol. Briefly, up to 1 x 10^7 cells were disrupted in RLT buffer and homogenized. Ethanol was added to the lysate and the sample was applied to the RNeasy Mini spin column and centrifuged. Three washing steps (one time buffer RW1, two times buffer RPE) were performed. The RNA was then eluted in 30 µl RNase-free water followed by direct reverse transcription.

3.2 Reverse transcription

3.2.1 Reverse transcription using random hexamer primers

The extracted viral RNA was reversely transcribed to single stranded cDNA using pd(N)6 random hexamer primers. For each reaction, 10 µl total RNA plus 10 µl PCR Mix were used. The RT reaction was performed in the thermocycler.

RT Mix for one reaction:

H_2O	4.1 µl
5x Puffer	4 µl
DTT 0.1M	0.1 µl
dNTP 25mM each	0.3 µl
N6-Primer (600 ng/µl)	0.5 µl
RNA Safe	0.5 µl
M-MLV-RT (200 U/µL)	0.5 µl
	10 µl

Incubation conditions:

25°C for 10 min
42°C for 30 min
96°C for 6 min
Cool down to 4°C

3.2.2 Reverse transcription using the SuperScript III First-Strand Synthesis System

The SuperScript III First-Strand Synthesis System was used prior to amplification of long PCR products for sequencing purposes. The SuperScript III Reverse transcriptase is a version of M-MLV RT that has been engineered to reduce RNase H activity and provide increased thermal stability.

Mix 1:
(for one reaction)

RT-HAN or N6 Primers	1µl
dNTP (10mM)	1 µl
RNA	8 µl
	10 µl

Mix 2:
(for one reaction)

10X RT Buffer	2 µl
25mM $MgCl_2$	4 µl
0.1M DTT	2 µl
RNaseOUT (40U/µl)	1 µl
Superscript III RT (200U/µl)	1 µl
	10 µl

Both mixes were prepared in separate tubes. First Mix 1 was prepared and incubated at 65°C for 5 min and then incubated on ice for at least one minute. Then Mix 2 was added and the tubes were placed in the thermocycler and the incubation was started.

Incubation conditions
48°C for 60 min
85°C for 5 min
on ice, at least one min
Add 1 µl RNase H to each reaction
37°C, 20 min
Cool down to 4°C

3.3. Polymerase chain reaction

3.3.1 Multiplex PCR

For detection and characterisation of putative reassorted viruses, a strain- and segment-specific multiplex PCR was established. Multiplex PCR is a variant of PCR which enables simultaneous amplification of several targets of interest in one reaction by using more than one primer pair.

This Multiplex PCR approach is composed of two PCR reactions. For each PCR, a set of four strain-and segment-specific primer pairs was designed. Primer set SK consists of primers specific for S, M and L Segment of SK/Aa and L segment of Slo/Af (primer set SK). The second primer set comprises primers specific for S, M and L segment of Slo/Af and L segment of SK/Aa (primer set Slo). The resulting PCR products differ in fragment size (Table 9).

	PCR Mix one reaction:	PCR program:	
		1 Cycle	95°C for 15 min
	25 µl PCR Mix	40 cycles	94°C for 30 seconds
	5 µl Primer Mix		52°C for 1 min
	1 µl Betaine Enhancer		72°C for 1 min
	14 µl H_2O		72°C for 6 min
Total	45 µl		cool down to 4–8°C and hold

PCR products were analysed and visualised by electrophoresis in 1 % agarose gel after 15 min of staining with ethidium bromide (1 µg/ml)

3.3.2 Detection of mycoplasma contamination by PCR

To detect potential contamination of the cell lines and virus stocks with mycoplasma, the Venor®*GeM* Mycoplasma PCR Detection Kit was used. Samples and protocol of PCR were prepared according to the instructions of the supplier. Briefly, 10 µl of cell culture supernatant from virus stock was taken and incubated for 10 min at 95°C and afterwards briefly centrifuged to pellet cellular debris. For each reaction 23 µl PCR mix was prepared and 2 µl of the sample was added.

The PCR master mix for one reaction:

Water	15.1 µl	PCR Programme:	
10x reaction buffer	2.5 µl	1 cycle	94°C for 2 min
Primer/nucleotide mix	2.5 µl	39 cycles	94°C for 30 seconds
Internal control	2.5 µl		55°C for 1 min
Taq polymerase	0.4 µl		72°C for 30 seconds
Total	23 µl		Cool down to 4–8°C

After thermal cycling, 5 µl of each sample mixed with loading buffer was loaded onto a 1.5% agarose gel and run for approximately 20 min at 100V.

3.3.3 RT-PCR for sequencing

To determine the reassorted genotype and exclude possible mutations, the S, M segments and the open reading frame (ORF) of the L segment of the parental virus Slo/Af as well as from the reassorted virus clones A6, B13, A35, A36 and B5 were sequenced. For parental SK/Aa, the L-ORF was sequenced since no sequence of the ORF of the L segment was available yet. Cell culture supernatants of infected Vero E6 cells were harvested eight days post infection and the RNA was extracted using the Viral RNA Mini Kit following the application protocol of the manufacturer. The RNA was reversely transcribed with the SuperScript III First-Strand Synthesis System from Invitrogen using random hexamer primers (3.2.2).

The S segment was obtained in a single PCR reaction using the single genus-specific primer RT-DOB for the parental viruses and for A6 and A36, as previously described by Klempa et al., 2005. For A35, B5 and B13, the complete S segment sequence was obtained by sequencing two overlapping PCR products (RT-DOB/D1162C, RT DOB/D955C and D113/RT DOB, D357F/RT DOB).

The M segment was obtained with nested PCR method. Primer pairs were chosen to amplify overlapping fragments to obtain the complete segment (M11/M3618,

G1NS/MD3643 for SK/AA and Slo/Af; MDTermini/M2029R, M11/M19990R, M1470/MD Termini, M1674/M3618, M1674/M3643 for A6 and A36; MD Termini/M2029R, M905F/M1990R, MD Termini/DOBM 1002R, M11/M2029R, M11/M1990R, M11/DOBM 1002R, M1674/M3618, M1851/M3618 for A35, B13 and B5 were used).

For the L segment of the parental viruses and of the reassortant progenies, eight primer pairs were chosen for amplification of overlapping fragments to obtain the whole segment. For parental SK/Aa, A6 and B13 following primer pairs were used: SK-L1-F/SK-L-R1 and SK-L-F1/SK-L3-R. For Slo/Af, A35, A36 and B5, the primer pairs Slo-L1-F/Slo-L-R1 and Slo-L-F1/Slo-L3-R were used. These long PCR products were then sequenced using the shotgun-based Long PCR Product Sequencing (LoPPS) protocol recently described (Emonet et al., 2007). Briefly, PCR products from RT-PCR are randomly sheared by sonication into approximately 700 bp DNA fragments. The protruding termini are repaired to create blunt ends using the DNA terminator End Repair Kit (Lucigen). In the next step, A-overhangs were added (A-addition Kit, Qiagen) at the 3´-end to allow TA cloning. Clones were randomly selected and prepared for sequencing as described in chapter 3.4.

3.3.4 Agarose gel electrophoresis

At least 5 µl cDNA-PCR products were mixed with 6x sample buffer and loaded on an agarose gel (1 %). The DNA was separated with 90-110 V and 300 mA for 30-60 min, depending on the size of the fragments. The gel was then stained in ethidium bromide solution (1 µg/ml) for 15 min and the DNA bands were visualised on a transilluminator.

3.4 Cloning and full length sequencing of S, M and L genomic segments

The amplified PCR products were either directly sequenced using respective primer pairs listed in Table 10 or cloned into the pSC-A PCR cloning vector using the StrataClone PCR Cloning Kit according to the instructions of the manufacturer. For sequencing of the plasmids, primers M13uni and M13rev were used (Table 10). Plasmids were then purified by the QIAGEN plasmid purification Mini Kit according to the instructions of the manufacturer. At least three recombinant plasmids were sequenced with the 3130 Genetic Analyzer using the Big dye Terminator V1.1 Kit. Briefly, the purified plasmids or PCR products were used as a template for sequencing reaction.

Sequencing reaction PCR	Programme		
4 µl Terminator Ready Mix	96°C	2min	
9 µl distilled water	96°C	10sec	x25
1 µl Primer (5µM)	50°C	5sec	
1 µl DNA (PCR 10 – 50ng)	60°C	4min	
15 µl	4°C	hold	

After sequencing reaction, the amplified products were transferred to an Eppendorf tube containing 80 µl of 75% isopropanol, mixed by vortexing and incubated for at least 15 minutes (maximum over night) at room temperature. Following two centrifugation steps, the isopropanol was carefully removed and the pellet was dried either at room temperature or for 1 minute at 90°C. Highly deionised formamide was then added, the tube was vortexed, briefly centrifuged, denatured at 90°C for three minutes, cooled on ice and then placed into the plate for sequencing.

3.5 Sequence comparison, phylogeny and recombination analysis

The overlapping nucleic acid sequences obtainedwere combined for analysis and edited with the aid of the SEQMAN program from the Lasergene software package (DNASTAR, Madison, Wis., USA). The sequence data were further analysed using the BioEdit software package (Hall, 1999). Sequences were aligned using CLUSTALW (Thompson et al., 1994) with default parameters.

To reconstruct maximum likelihood (ML) phylogenetic trees, quartet puzzling using the TREE-PUZZLE package was applied (Schmidt et al., 2002; Strimmer and vonHaeseler, 1997). As evolutionary model for the reconstructions the Tamura-Nei model was used and missing parameters were reconstructed from the datasets. The values at the tree branches are the resulting PUZZLE support values, if not otherwise stated. Resulting evolutionary trees were then visualised using TreeView v.1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Similarity plot analysis was performed with Stuart Ray´s SimPlot 3.2 with default parameters (Lole et al., 1999). For the analyses, a window of 200 nucleotides (nt) and a step size of 20 nt were used.

3.6 Virus stock propagation

Hantavirus stocks were prepared on VeroE6 cells cultivated in 75 cm^2 cell culture flaks. The cells were inoculated at the multiplicity of infection (MOI) 0.1. After one hour´s incubation at 37°C in 5% CO_2 humidified atmosphere, 15 ml medium was added and the flasks were incubated as described before. After seven days, cell culture supernatant was harvested, centrifuged to remove cell debris, aliquoted and stored at -80°C.

All virus stocks were determined to be free of mycoplasma as tested by VenorGeM mycoplasma detection kit (see 3.3.2)

3.7 Generation of reassortants

For generation of reassorted viruses, VeroE6 cells grown in 25 cm^2 cell culture flasks ($1x10^6$ cells/flask) were co-infected with the parental viruses SK/Aa and Slo/Af. To examine whether the amount of virus has an influence of the reassortment process, different multiplicity of infections (MOI) of both viruses were used for the infection. After adsorption of the inoculum for 90 min at 37°C in 5% CO_2 humidified atmosphere, 7 ml medium (supplemented with 10% FCS) was added per flask. After nine days of incubation, the cell culture supernatant was collected and centrifuged to remove cellular debris. The virus output from the experiment was determined as described in chapter 3.8. The supernatant was aliquoted and stored at –80°C.

3.8 Titration of hantaviruses

To determine the virus titers of the viral stocks, the modified chemiluminescent focus assay described by Heider et al. (2001) was used. Briefly, confluent Vero E6 cells grown in 6-well plates were infected with virus diluted in 1:10 steps (200µl/well). After virus adsorption for one hour at 37 °C in a humidified 5% CO_2 atmosphere, the cells were overlaid (2.5 ml overlay per well) with a 1:1 mixture of 2.4% Avicel and basal Eagle´s medium (2x BME) supplemented with 5% FCS, 2.5% Hepes and antibiotics. Plates were then incubated for 7–10 days (dependent on the virus strain) under the conditions described above.

Afterwards, the overlay was removed by gently pouring of washing buffer (PBS supplemented with 0.15% Tween-20) onto the overlay. By flipping the plate, the overlay was discarded. The cells were carefully washed twice with washing buffer, then fixed with methanol for 8 min, allowed to dry and again washed two times.

1 ml of corresponding rabbit polyclonal antiserum was added to each well, diluted in PBS containing 10% FCS, and incubated for 1 h at 37 °C.

Cell were washed five times and 1 ml of goat anti-rabbit IgG conjugated with horseradish peroxidase diluted 1:1000 in PBS supplemented with 10 % FCS was added per well and incubated for 1 hour (h) at 37 °C.

Following five additional washing steps, 0.5 ml/well of the chemiluminescence substrate, Supersignal West Dura, diluted 1:5 in water, was added. The plates were evaluated in DIANA Chemiluminescence System (Raytest, Straubenhardt, Germany).

Evaluation of the titers: The focus forming units (FFU) of each well were counted and the titers were calculated per ml.

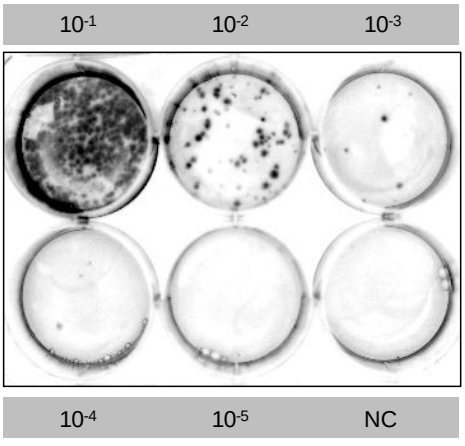

Figure 5: Chemiluminescent detection of hantaviral FFUs: Titration of Slo/Af in a 6-well plate in ten-fold dilutions

3.9 Focus purification assay

Single viral clones were isolated using the recently described focus purification assay (Rang et al., 2006). VeroE6 cells, grown in 10 cm petri dishes, were infected by different dilutions of hantaviruses and overlaid with a 1:1 mixture of 1.2% agarose and basal Eagle´s medium (2x BME) supplemented with 5% FCS, 2.5% hepes and antibiotics. Plates were then incubated for 7–10 days. (Figure 6 A). After the incubation period the overlay was engrailed in position 12 o´clock, carefully turned upside down and stored for isolation of the virus diffused into it (Figure 6 B). Cells were fixed with methanol (Figure 6 C). Viral antigen-containing foci were detected using the chemiluminescent focus assay (Heider et al., 2001) described above (Figure 6 D).

To detect the relative location of the foci, a hard copy (Figure 6 E) of the processed image was held under a petri dish containing the agarose overlay and used as a map to trace and pick individual virus clones diffused into the corresponding region of the overlay (Figure 6 F). Several clones were picked with a 1 ml pipette tip (Figure 6 G). The picked material was thoroughly mixed in 200 µl medium. Each clone was used for re-infection of one well of a 24-well plate (Figure 6 H).

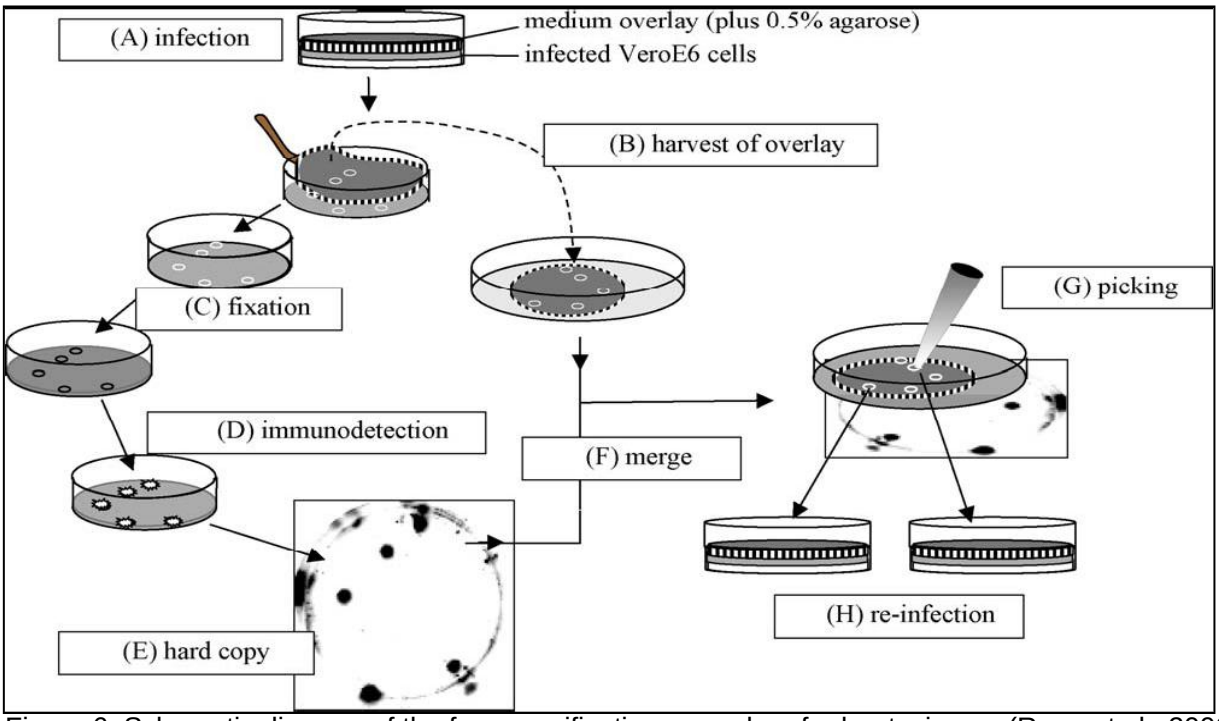

Figure 6: Schematic diagram of the focus purification procedure for hantaviruses (Rang et al., 2006)

3.10 Screening for reassortants

For investigation of reassorted viruses, single viral clones were isolated using the Focus purification assay as described above. For each clone, the RNA was extracted from supernatant using the Viral RNA Mini Kit (Qiagen, Hilden, Germany). The isolated RNA was reversely transcribed as described in chapter 3.2.1 and then further analysed by Multiplex PCR (chapter 3.3.1).

3.11 Protein chemistry

3.11.1 MxA Expression

The expression of the MxA protein was examined in A549 and HuH7 cells. Cells were grown in 12-well plates and infected with MOI 1 of the respective DOBV strain. Cells infected with PHV were taken as a positive control. Uninfected cells were taken as a negative control. Plates were incubated at 37°C in 5% CO_2 humidified atmosphere. At time points one, two, three and four days post infection (d p.i.), samples were taken for each virus. Briefly, the medium was removed and cells were carefully washed with PBS. After removing the PBS, lysis buffer was added to the cells. The plates were incubated for 15 min at 37°C. Afterwards, the lysate was transferred to 0.5 ml tube and stored at -20°C until analysis by western blot.

3.11.2 Western blot

Cell extracts were prepared by lysing the cells with 2x SDS sample buffer. To remove cell debris, the lysate was centrifuged and afterwards boiled at 95°C for denaturing of the proteins. For separation of the proteins, samples were loaded on a 10% polyacrylamide gel. The gel was run at 25 mA/gel.

Table 11: Composition of SDS Gels for Western Blot Analysis

	Resolving gel 10%	**Stacking gel 5%**
30% Acrylamid/Bis (29:1)	3.3 ml	0.83 ml
1.5M tric HCl (pH8.8)	2.5 ml	-
0.5M Tris HCL (pH 6.8)	-	1.25 ml
H2O	4 ml	2.8 ml
10% SDS	100 µl	50 µl
10% APS	100 µl	50 µl
TEMED	6 µl	6 µl

Following electrophoretic separation, proteins were transferred onto methylcellulose membrane by semi-dry blotting method at 75 mA/gel. The resolving gel was cut from the stacking gel. Three pieces of Whatman paper were soaked with blotting buffer and placed onto the semi-dry electroblotting device. The gel was placed onto the Whatman paper. The nitrocellulose membrane was equilibrated in the semidry blotting buffer and placed onto the gel. Finally three additional Whatman papers were put onto the membrane. The transfer was performed for one hour at 75 mA/gel. After the transfer, the efficiency was checked by staining the membranes with Ponceau red. Then the blots were blocked for 1 h at room temperature in TBST containing 5% milk powder. Specific primary antibodies, diluted in TBST containing 2% milk powder, were added to the blots and incubated for 1 hour to 12 hours at 4°C. The membranes were then washed five times with TBST, followed by incubation with appropriate secondary antibodies conjugated with horseradish peroxidase for 1 hour at room temperature. After five final washing steps, detection of the stained proteins was performed by adding chemiluminescent substrate (SuperSignal West Dura Extended Duration Substrate) to the membranes. The proteins were visualised by exposure on a CCD camera.

3.12 Luciferase Reporter Gene Assay

Activation of the interferon-stimulated response element (ISRE) was measured for phenotyping the parental DOBV strains and the reassorted viruses. For this purpose, Huh7 cells were seeded onto 48 well plates. The cells were transfected using an ISRE-controlled luciferase expression construct. Sufficient amount of the transfection reagent Lipofectamin was carefully mixed with Opti-MEM by pipetting and incubated for 15 min at room temperature. DNA (500 ng) was mixed with the appropriate amount of Opti-MEM. After 15 minutes´ incubation both solutions were mixed together and again incubated for 15 minutes at room temperature. Then 100 µl of the transfection mix was added in each well. Six hours after transfection, the cells were infected in different concentrations (MOIs 0.05, 0.25 and 1) with the respective viruses. Cells were incubated at 37°C in 5% CO_2 humidified atmosphere for 24 hours. After that incubation time, the cells were harvested and assayed with the luciferase reporter gene assay. Briefly, the media was removed and cells were carefully washed with PBS. For lysis of the cells, sufficient amount of reaction buffer was added to each well and incubated for approximately five minutes at room temperature. Samples were collected in tubes and transferred to a microplate. To each sample substrate reagent, was added and assayed with a luminometer.

4. Results

4.1 Establishment of strain- and segment-specific Multiplex PCR as a reassortment screening assay

For detection of reassorted viruses in a single-tube reaction, a multiplex PCR assay with four strain- and segment-specific primer pairs (Table 9) was established. Three primer pairs were selected to amplify three products of different size representing S, M and L genomic segment of SK/Aa. As an internal control, a fourth primer pair was included amplifying a part of the Slo/Af L segment. It makes it possible to distinguish between a negative PCR reaction as a result of missing viral cDNA and detection of the parental Slo/Af virus (which would also give no PCR reaction if only SK/Aa-specific primers were used). This system allows to differentiate the parental strains (three products representing parts of all three segments in the case of SK/Aa, one L segment-specific product in the case of Slo/Af) and the putative reassortants (any of the three SK/Aa specific products missing or the Slo/Af L segment-specific product in combination with SK/Aa S or M segment-specific products). As a confirmatory assay, we established analogous multiplex PCR assay with three Slo/Af primer pairs and one SK/Aa L segment specific primer pair (Table 9).

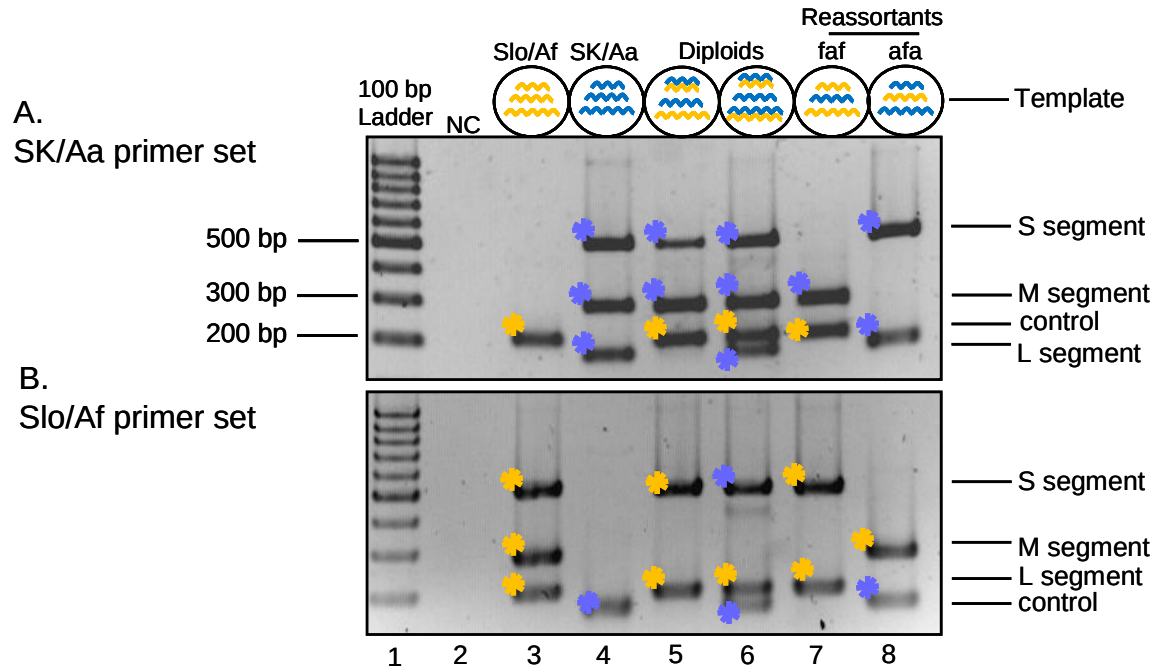

Figure 7: Representative image of a multiplex PCR profile. Fragments of the parental and reassorted viruses were amplified by PCR using SK/Aa-specific primer set (A) and Slo/Af-specific primer set (B). Lane 1 and 2 show the 100bp ladder and negative control, respectively. PCR patterns of the parental viruses SK/Aa and Slo/Af are shown in the next two lanes (3 and 4). In lanes 5 and 6 PCR patterns for diploid viruses and in the last two lanes the PCR patterns for reassortants are shown. For explanation of designations faf and afa, see chapter. 4.2.3.

Testing of viral clones by both assays made it possible to detect and unambiguously distinguish the parental strains, all possible reassortants, as well as the diploid viruses (containing particular genomic segments of both parental virus strains) (Figure 7).

4.2 Generation and screening of reassorted viral clones

4.2.1 Co-infection of VeroE6 cells

For generation of reassorted viruses, VeroE6 cells were co-infected with the parental viruses SK/Aa and Slo/Af. To examine whether the ratio of viruses has an influence on the reassortment process, different MOIs of both viruses were used for the infection (Table 12). The supernatant was collected nine days after infection and the titer of each co-infection experiment was determined by virus titration as described in chapter 3.8.

Table 12: Co-infection experiments for generating reassorted viruses. Indicated are the MOIs of the parental viruses used for co-infection and the titer after harvesting of the supernatant of each experiment.

Experiment	MOI of viruses		Virus titer after co-fection (FFU/ml)
	SK/Aa	Slo/Af	
A	0.25 :	0.25	$1*10^5$
B	0.45 :	0.05	$2*10^5$
C	0.05 :	0.45	$1*10^5$

4.2.2 Screening of viral clones

For isolation of single viral clones, VeroE6 cells grown in 10 cm Petri dishes were infected with supernatant from co-infection experiments (4.2.1). According to their titers, the stocks were diluted to approximately 20 – 40 focus forming units (FFU) per 10 cm Petri dish. Single viral clones were isolated using the focus purification method (Rang et al., 2006) followed by RNA extraction, reverse transcription and multiplex PCR.

Table 13: Genomic analysis of isolated virus clones. Indicated are the numbers of parental, reassortant, and diploid virus clones detected in three independent dual infection experiments.

Experiment	MOI of viruses		SK/Aa	Slo/Af	Reassortant Genotype		Diploid	Total
	SK/Aa	Slo/Af			$a_S f_M a_L$*	$f_S a_M f_L$*		
A	0.25 :	0.25	9	14	1	44	36	104
B	0.45 :	0.05	25	1	3	13	8	50
C	0.05 :	0.45	0	28	0	4	21	53
Total	-		34	43	4	61	65	207

*a : SK/A**a** parental origin; f : Slo/A**f** parental origin; S, M and L indicating the respective genome segment

In total, 207 viral clones were isolated and examined for reassortment events by multiplex PCR (Table 13). A reassorted genotype was detected in 65 clones (31%). The highest number of reassortants (45/104) was detected when an equal ratio of parental viruses was used. When the amount of the parental SK/Aa virus was 9-fold higher than Slo/Af, 16 out of 50 analysed clones were identified as reassortants. 25 clones were identified as parental SK/Aa in comparison with one parental Slo/Af clone. When the ratio of the parental viruses was 9:1 for Slo/Af, the proportion of reassortants was substantionally lower (4/53) and no parental SK/Aa clones in comparison to 28 Slo/Af clones were detected. Altogether, 37% (77/207) of the isolated clones represent the parental viruses, 34 clones were SK/Aa and 43 Slo/Af. Furthermore, 65 (31%) of 207 clones were identified as diploid viruses, containing both versions of the respective genome segment (see chapter 4.2.4).

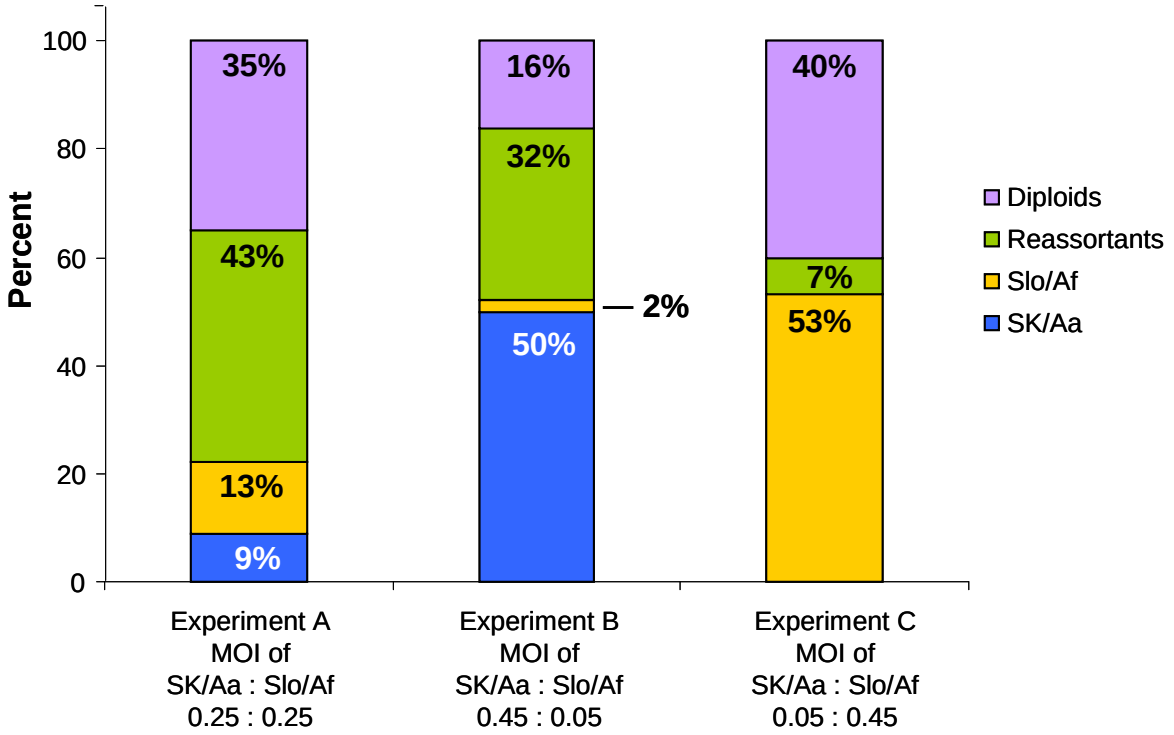

Figure 8: Percental allocation of parental viruses, reassortants and diploids detected in the three co-infection experiments. In total, 207 viral clones were isolated. From experiment A 104 clones were isolated, from Experiment B 50 and from Experiment C 53 single viral clones were isolated.

One hundred and four viral clones were isolated from co-infection experiment A (MOI 0.25 for both parental viruses). Nine percent of the viral clones were identified as SK/Aa and 13% as Slo/Af. Fourth three percent of the clones were identified as reassortants and 35% as diploids (Figure 8).

From experiment set up B (MOI 0.45 SK/Aa vs. MOI 0.05 Slof/Af), 50 viral clones were isolated. 50% of them were examined as SK/Aa in contrast to 2% Slo/Af genotype. Reassortants were identified in 32% of the clones in comparison to 16% diploid viral clones (Figure 8).

Fifty three viral clones were isolated from experiment C (MOI 0.05 SK/Aa vs. MOI 0.45 Slo/Af). From this experiment, none of the isolated clones were identified as parental SK/Aa, but 52% showed the parental Slo/Af genotype pattern. Seven percent were examined as reassortants in contrast to 40% diploids (Figure 8).

4.2.3 Two stable reassortment patterns detected

In 65 identified reassortant virus clones, only two out of six hypothetical reassortment patterns were detected. In both cases, the genomic M segment was exclusively exchanged.

Interestingly, genotype designated as afa (containing the S and L segment of SK/A**a** and the M segment of Slo/A**f**) was found only in 4 clones, whereas in 61 clones the S and L segments of Slo/A**f** and the M segment of SK/A**a** (genotype faf) were present (Figure 9).

For further characterisation and functional analysis, at least two clones of each reassortment pattern were chosen. For the afa pattern, clones named A6 and B13, and for the faf pattern clones A35, A36 and B5 were further analysed.

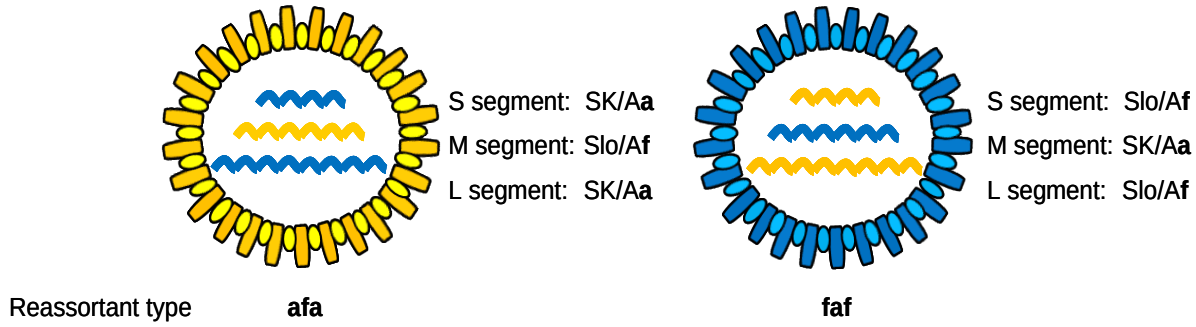

Figure 9: Schematic diagram of the two stable reassortment scenarios. Reassortant types were designated as afa and faf. The a indicates the parental SK/A**a** origin and f indicates the origin of parental Slo/A**f**.

4.2.4 Detection of viruses with diploid genotype

In addition to the reassorted viruses, viral clones with diploid genotypes were detected (65 out of 207 isolated clones). These viruses contained both versions of the particular genome segments. In this study seven different diploid genotype patterns were detected (Table 14). Out of 65 diploids, 52 showed the diploid M segment (of SK/Aa and Slo/Af origin) with S and L segment origin of Slo/Af. In contrast one clone out of 65 was examined with diploid M genome pattern and S and L segments of SK/Aa. Seven clones were identified to be diploid in the S and L segments while the M segment was of parental SK/Aa origin. Two clones contained two L segments. Diploid genome pattern for all three segments, the S and M segments or just the S segment was detected in one clone respectively.

Table 14: Genomic characterisation of diploids. Indicated are numbers of diploid virus clones and observed genotypes after dual infections.

Exp.	MOI of viruses		Diploid Genotype							Total
	SK/Aa	Slo/Af	$af_Sa_Maf_L$	$f_Saf_Mf_L$	$a_Saf_Ma_L$	$a_Sa_Maf_L$	$af_Saf_Maf_L$	$af_Saf_Mf_L$	$af_Sa_Mf_L$	
A	**0.25**	**0.25**	4	29	1	1	0	1	0	36
B	**0.45**	**0.05**	3	2	0	1	1	0	1	8
C	**0.05**	**0.45**	0	21	0	0	0	0	0	21
Total	**-**	**-**	7	52	1	2	1	1	1	65

a – SK/Aa parental origin; f – Slo/Af parental origin; S, M and L indicating the respective genome segment

Nine detected diploids were passaged on cell culture and then three times focus purified. After focus purification, all nine clones lost their diploid genotype, yielding in most cases parental and just in one case the reassortant faf genotype (Table 15).

Table 15: Detected Genotypes of nine diploids. Indicated are the diploid genotypes after isolation, the number of clones and the genotypes after additional plaque purification steps.

Diploid genotype	No. of clones passaged	Genotypes finally found
$af_Sa_Maf_L$*	4	a a a
$a_Sa_Maf_L$	2	a a a
$a_Saf_Ma_L$	1	a a a
$af_Sa_Mf_L$	1	f a f
$af_Saf_Maf_L$	1	a a a

*a – SK/Aa parental origin; f – Slo/Af parental origin; S, M and L indicating the respective genome segment

4.3 Nucleotide sequence analyses

Sequence analyses were performed to confirm the reassortant pattern observed by MP-PCR (chapter 3.3.1) and to study whether particular mutations accumulated during the reassortment process. Complete sequences of the S and M genomic segments and complete L segment-ORF sequences of all five clones were determined and compared with complete sequences of the parental strains. Moreover, complete L segment sequences of both parental strains were determined. For the Slo/Af strain used here (designated as Slo/Af-BER), S and M segments were also re-sequenced because the sequences available in GenBank were released in 1994 and could substantially differ from the sequences of the current virus stock in our laboratory. The sequences obtained of Slo/Af and the reassortants were compared with sequences available in GenBank. Accession numbers for Slo/Aa S segment: L41916 and M segment: L33685; accession numbers for SK/Aa S segment: AY961615 and M segment: AY961616. All differences detected in the sequences are described in chapter 4.3.1 and summarised in Table 16 and Table 17.

4.3.1 Nucleotide sequence analyses of Slo/Af and the reassorted viruses

The complete S segment sequences of the clones A6 and B13 were found to be of the same length as the parental SK/Aa sequence (1,697 nt in length containing a single open reading frame [ORF; nt 36 to 1,325] encoding the viral N protein of 429 aa). B13 was completely identical to SK/Aa while a single, silent mutation was found in the S segment sequence of A6 (G965C). In the case of Slo/Af we found differences also between the published sequence and our Slo/Af-BER sequence; two consecutive mutations (T1035G, G1036T) and two insertions/deletions in the 3'-NCR (nt positions 1345 and 1352). The complete S segment sequences of the clones A35 and B5 as well as Slo/Af-BER were found to be 1,672 nt long and identical to each other. Comparison of the A36 S segment sequence revealed a 24 nt deletion in the 3´NCR region of A36 (positions 1360-1384 of the Slo/Af-BER nt sequence) and one additional mutation in the 3'-NCR (G1431A).

For the M segment, sequences of A35 and B5 clones were found to be identical to the SK/Aa sequence (3,643 nt in length containing a single ORF [nt 41-3445] which encodes the putative glycoprotein precursor of 1,135 aa). A36 sequence differed from the SK/Aa sequence just by one silent mutation in the ORF (T2809C) and one mutation in the 3'-NCR. Several differences were again found between the published Slo/Af

sequence and our Slo/Af-BER. Three mutations (A2777G, C2861T, A3181T) led to amino acid exchanges R913G, R941W, and Q1047H, while one mutation (T3043A) was silent. Moreover, a deletion of three nucleotides at the position 3381 was found in Slo/Af when compared with Slo/Af-BER. In all these positions, both clones A6 and B13 resembled the Slo/Af-BER sequence. B13 differed from Slo/Af-BER just by one mutation (A2598T) leading to amino acid exchange E853V. In addition A6 had one non-silent mutation (T546C causing aa exchange V169A) and one silent mutation (A1471C) in the ORF and two changes in the 3'-NCR region (insertion/deletion at positions 3608 and T3613C).

The L segment ORF encoding viral RNA polymerase was found to be 6,453 nt long (2,151 aa) in both parental strains as well as all five reassortant clones. When comparing SK/Aa with A6 and B13 sequences, eight nucleotide differences were found. Three of them (C859T, A973T, G2479A) cause amino acid exchanges H287Y, I325F, and D827N. All three were found in B13 sequence while A6 contained only the D827N exchange. Interestingly, all five silent mutations (A585G, G660A, C859T, C2322T, C2325T, A2913T) were found in both A6 and B13 sequences. Comparison of the parental Slo/Af-BER L segment sequence with the A35, A36, and B5 sequences revealed altogether 11 mutations, five causing amino acid exchange and six being silent. Most differences were found in the A35 sequence containing four amino acid changing mutations (C1118T, A2411G, A2621G, and G5429A leading to amino acid exchanges P373L, K804R, K874R, and C1810Y, respectively) and five silent mutations (A2649G, G2718A, C3933T, A5316G, and A5352G). Two amino acid changing mutations (C1118T and G6322A leading to amino acid changes P373L and E2108K, respectively) and three silent mutations (T1464C, G2718A, and C3933T) were found in the A36 sequence. B5 sequence showed only three mutations, one non-synonymous (C1118T leading to P373L) and two synonymous (G2718A and C3933T).

Furthermore, similarity plots were prepared for the sequences obtained, which confirmed that no homologous recombination occurred during the dual infections. Maximum-likelihood trees constructed for all three segments confirmed represent the afa genotype for A6 and B13 and faf pattern for A35, A36 and B5 clones (Figure 11).

Table 16: Summary of mutations observed in reassortant clones. Sequences of the reassortants were compared with sequences of parental strains. Mutations leading to amino acid exchanges (shown in brackets) are in bold, mutations in the non-coding regions are shown in italics.

	Mutations in comparison with		
Virus clone	**$S_{SK/Aa}$**	**$M_{Slo/Af\text{-}BER}$**	**$L_{SK/Aa}$**
A6	G965C	**T546C (V169A)** **A2598T (E853V)** A1471C *in./del.* 3608* *T3613C*	**G2479A (D827N)** A585G, G660A C2322T C2325T, A2913T
B13	-	**A2598T (E853V)**	**C859T (H287Y)** **A973T (I325F)** **G2479A (D827N)** A585G, G660A C2322T C2325T, A2913T
Virus clone	**$S_{Slo/Af\text{-}BER}$**	**$M_{SK/Aa}$**	**$L_{Slo/Af\text{-}BER}$**
A35	-	-	**C1118T (P373L)** **A2411G (K804R)** **A2621G (K874R)** **G5429A (C1810Y)** A2649G, G2718A, C3933T, A5316G, A5352G
A36	*deletion 1360-1383* *G1431A*	T2809C *G3615A*	**C1118T (P373L)** **G6322A (E2107K)** T1464C, G2718A, C3933T
B5	-	-	**C1118T (P373L)** G2718A, C3933T

- = no mutation detected; in/del = insertion/deletion

Table 17: Summary of mutations observed in original Slo/Af sequences available in GenBank (Acc. No. L41916 for the S segment and L33685 for the M segment) in comparison to the re-sequenced So/Af-BER. Nucleotide sequence from Slo/Af-BER used in this study was compared with the sequence. Mutations leading to amino acid exchanges (shown in brackets) are in bold, mutations in the non-coding regions are shown in italics.

	$S_{Slo/Af\text{-}BER}$	**$M_{Slo/Af\text{-}BER}$**	**$L_{Slo/Af\text{-}BER}$**
Slo/Af	**G1036T (V334C)** T1035G *in./del. 1345, 1352*	**A2777G (R913G)** **C2861T (R941W)** **A3181T (Q1047H)** T3043A in./del.* 3381-3383	n.a

n.a. = not available; in/del = insertion deletion

4.3.2 Molecular phylogenetic analyses of the parental viruses and the reassortants

Maximum-likelihood (ML) trees constructed for all three segments confirmed the afa genotype for A6 and B13 and faf pattern for A35, A36 and B5 clones. Clones A6 and B13 clustered with SK/Aa sequences in the S and L segment trees. In the M segment tree, they cluster with Slo/Af sequence. On the other hand, clones A35, A36 B5 clustered with Slo/Af sequences in the S and L segment trees but not in the M segment tree (Figure 10).

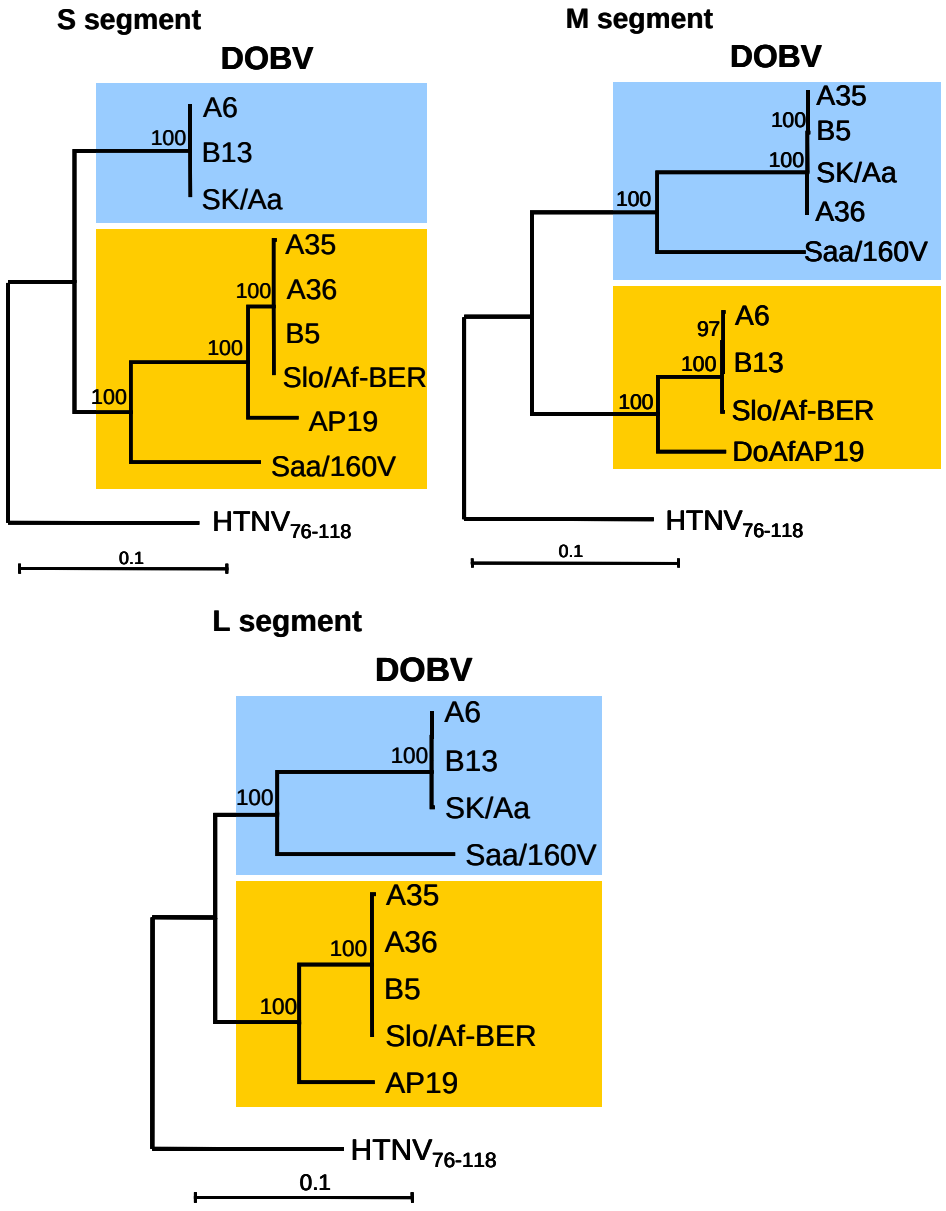

Figure 10: ML phylogenetic trees of DOBV strains SK/Aa, Slo/Af and the reassortants afa and faf based on complete S and M segment nucleotide sequences and L segment ORF sequence. DOBV lineages are marked by coloured boxes: blue, DOBV-Aa; yellow DOBV-Af. The trees were constructed using TREE-PUZZLE software package with Tamura-Nei evolutionary model, the values at the tree branches are the puzzle support values. The scale bar indicates an evolutionary distance of 0.1 substitutions per position in the sequence. $HTNV_{76-118}$, Hantaan virus strain 76-118.

4.3.3 Similarity plots

Similarity plots were prepared for the complete sequences obtained. No changes in the similarity patterns were observed, confirming that no homologous recombination occurred in the analysed reassortant clones (Figure 11).

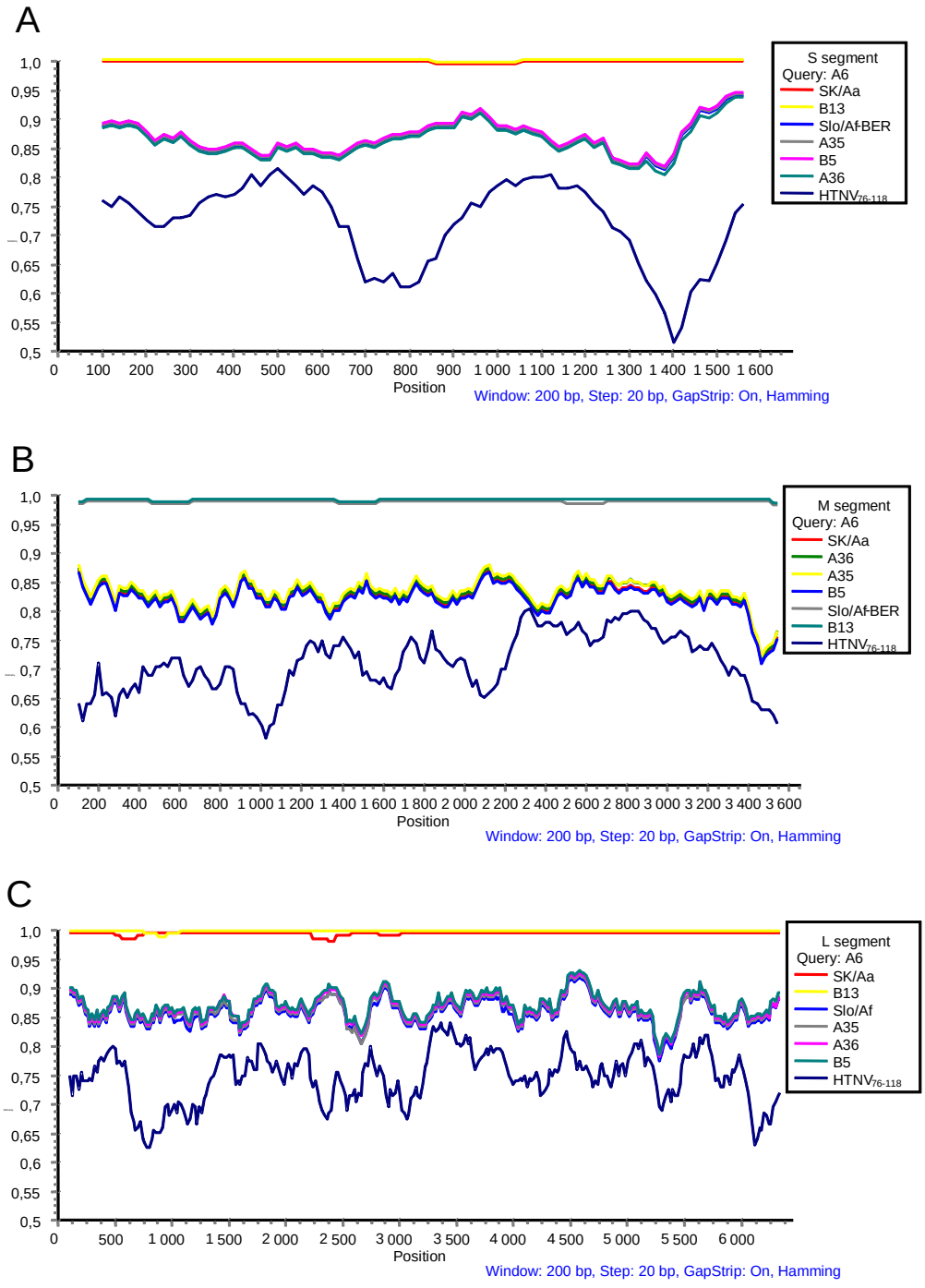

Figure 11: Similarity plots of SK/Aa, Slo/Af and the reassorted progenies. Sequences from SK/Aa, Slof/Af and the reassortants were compared against the sequences of the S, M and L segments of the reassorted clone A6. The plots were prepared using the SimPlot software program with default parameters.

4.4 Functional analyses of the parental viruses and their reassorted progeny

4.4.1 Growth kinetics in A549 wild-type and A549 RIG-I knockdown cells

For characterising the parental viruses and their reassorted progenies, growth kinetics experiments in different cell lines were carried out. Virus replication was examined in A549 cells and in A549 cells stably transfected with a shRNA expression construct targeting RIG-I (ΔRIG-I knockdown cells). Both cell lines were kindly provided by Markus Matthäi (Robert-Koch Institute, Berlin). To prove the RIG-I knockdown, the amount of RIG-I in the cell lines was examined by Western blot analysis using a RIG-I specific antibody. In A549 cells a strong expression of RIG-I was detected after treatment with type I IFN. In ΔRIG-I knockdown cells, no expression of RIG-I was detected (Figure 12).

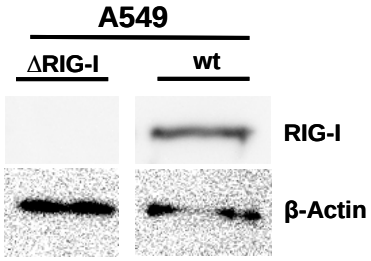

Figure 12: Western blot of RIG-I expression in A549 and A549 ΔRIG-I knockdown cells. Lysates were prepared from confluent cells treated with type I IFN (500 U/ml) for 24 hours.

For the growth kinetic experiment, A549 and the ΔRIG-I knockdown cells were grown in 6-well plates and infected with parental viruses SK/Aa and Slo/Af (both at MOI 1), as well as with the reassortants A6 (afa) and A36 (faf) (both MOI 0.5). Cell culture supernatant was harvested every day for seven d p.i.. To examine the virus replication, the collected supernatants were titrated on VeroE6 cells.

Three independent kinetic experiments for the parental viruses and two for the reassortants were done. Representative results are shown.

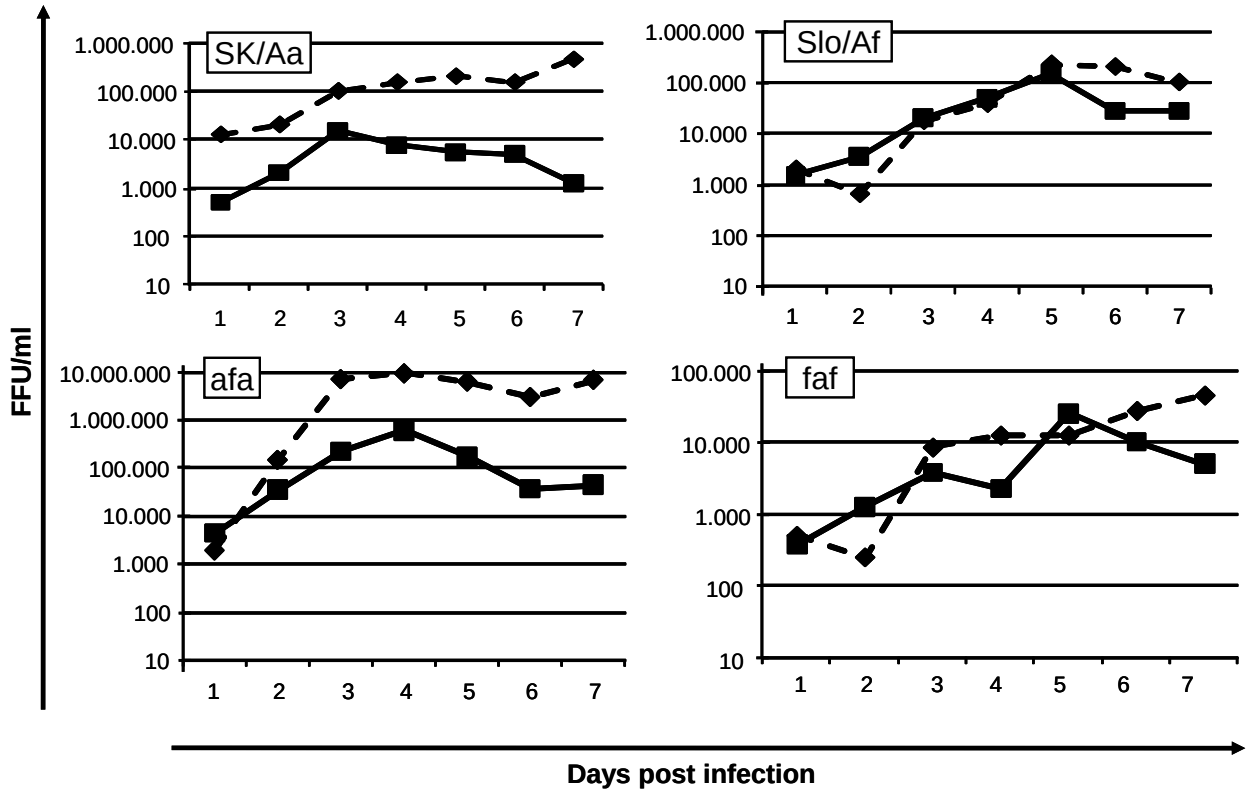

Figure 13: Growth curves of DOBV in A549 and ΔRIG-I A549 cells. A549 (black line) and ΔRIG-I knockdown cells (dashed line) were infected with MOI 1 for SK/Aa and Slo/Af and MOI 0.5 for reassortants afa (clone A6) and faf (clone A36). Supernatant was harvested at indicated time points after infection and titrated in Vero cells.

In ΔRIG-I cells, the titer of SK/Aa increased from day one to day seven after infection. In A549 wild type cells, the titer for SK/Aa increased until at least seven days post infection (Figure 13). Interestingly, SK/Aa replicated to more than 10-fold higher titers in RIG-I knockdown cells compared to replication in RIG-I competent cells. In contrast, Slo/Af replicated in both cell lines to titers similar to those of SK/Aa in the RIG-I cells. This result suggests that RIG-I modulated the replication of SK/Aa, but not of Slo/Af.

The replication of the reassortant afa (viral clone A6) was similar to the results of SK/Aa. The reassortant afa replicated to more then 10-fold higher titers in ΔRIG-I cells compared to the replication in RIG-I competent A549 cells. In contrast, reassortant faf replicated to lower titers, and is consistent with the kinetics observed for Slo/Af. The reassortant also replicated with the same efficiency in both cell types, irrespective of whether RIG-I was expressed or not. These results could indicate that the exchange of the M segment did not alter the characteristic RIG-I associated replication pattern, as long as S and L segments are conducted from the respective parental virus.

4.5 Expression of MxA protein induced viral infection

Recently, it was shown that pathogenic and non-pathogenic hantaviruses differentially induce the innate immune response. The interferon-inducible MxA protein was used to monitor hantavirus-mediated induction of the interferon system. The pathogenic Hantaan virus (HTNV) shows a late induction of MxA in A549 cells and no induction in HuH7 cells. In contrast, the non-pathogenic Prospect Hill virus shows an early induction in both cell lines (Handke et al., 2009). For phenotyping the DOBV parental viruses and reassortants (clones A6 and A35), A549 and HuH7 cells were infected with both parental viruses and their reassorted progeny with MOI 1. Cells were harvested at indicated days post infection and analysed by Western blot. Hantaviral N protein was detected as a measure of virus infection. β-actin was detected as loading control of the Western blot assay. As a positive control, cells were infected with PHV and uninfected cells were taken as a negative control. Data shown here are representative for two experiments carried out independently.

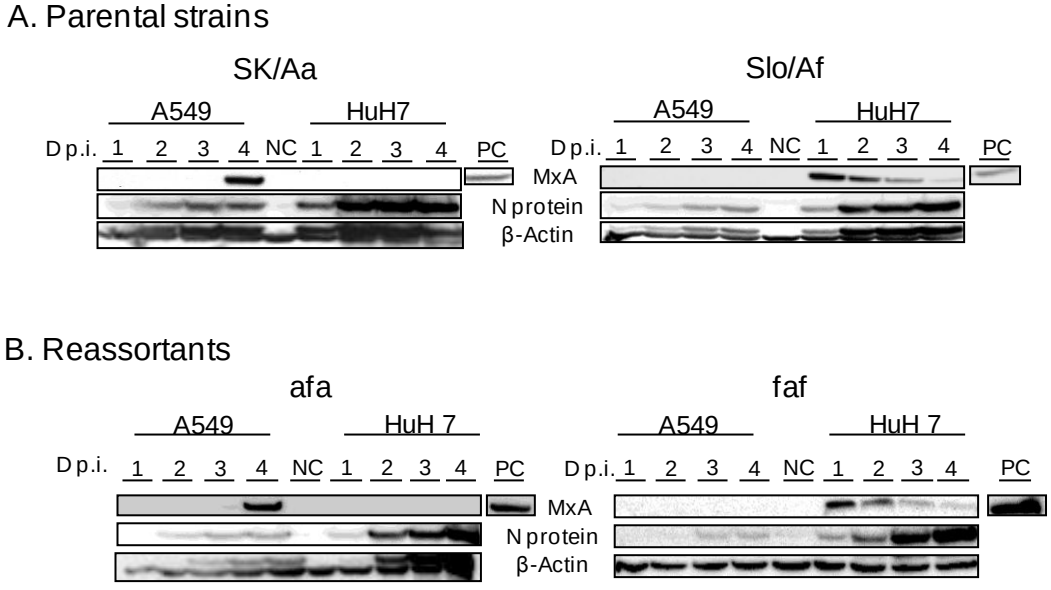

Figure 14: Expression of MxA in A549 and HuH7 cells. Western blot for the induction of MxA protein in A549 and HuH7 cells infected with the parental strains SK/Aa, Slo/Af (A) and reassortants afa (clone A6) and faf (clone A35) (B)

In A549 cells infected with SK/Aa, the antiviral MxA protein was induced late after infection. On day four post infection, a strong signal was detected, whereas in HuH7 cells infected with SK/Aa, no induction of MxA was detected. In A549 cells infected with Slo/Af, no expression of MxA was detectable (Figure 14). In Slo/Af infected HuH7 cells, MxA expression initiated early after infection and decreased steadily. Expression of N protein increased during the following days in both cell lines. Analogous MxA expression patterns were detected for the reassortant viruses. DOBV-afa induced the same MxA expression pattern as SK/Aa, with late induction of MxA in A549 cells and no induction in HuH7 cells. The induction of MxA by DOBV-faf resembled Slo/Af, no induction of MxA in A549 cells and early induction with decreasing concentration in HuH7 cells were observed. Also for the reassortants, the expression of the N protein increased during the following days in both cell lines. These results suggest that the M segment is not decisive for the characteristic MxA induction pattern.

4.6 Activation of the interferon stimulated response element

To further investigate the influence of DOBV members on the innate immune response, the activation of the interferon stimulated response element (ISRE) was examined. HuH7 cells were transfected with a luciferase expression plasmid which is controlled by an ISRE. Six hours after transfection the cells were infected with parental SK/Aa and Slo/Af as well as with the reassorted viruses afa and faf in different concentrations (MOIs of 1, 0.25 and 0.05). Twenty four hours post infection cells were assayed using the Luciferase Reporter Gene Assay (Roche). Data shown in this graph are representative for three independent experiments.

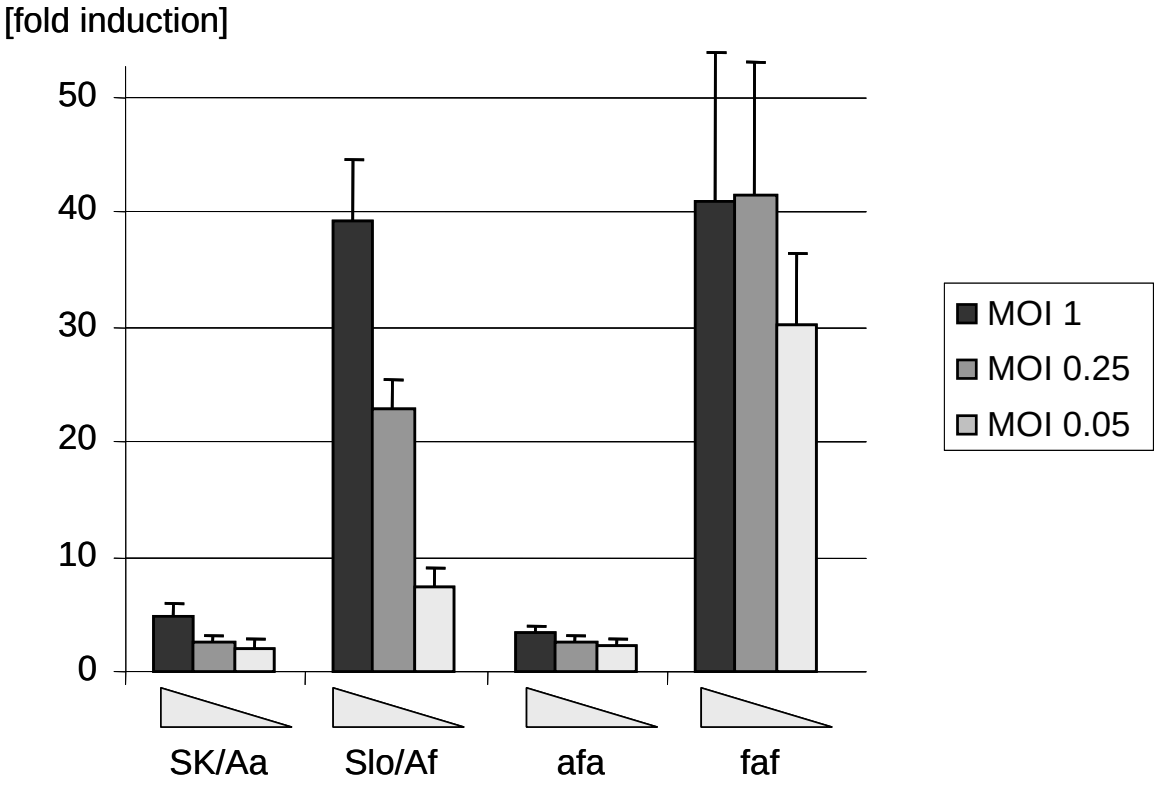

Figure 15: Induction of the Interferon stimulated response element (ISRE) by SK/Aa, Slo/Aa, DOBV-afa and DOBV-faf in HuH7 cells. Activation of ISRE is shown as factor of luciferase activity in samples from infected compared to not infected cells. Columns and T-bars represent mean and standard deviations, respectively (n=3).

As shown in Figure 15, SK/Aa activated the transfected ISRE. At MOI 1, five-fold higher signals were measured in SK/Aa infected cells in comparison to uninfected cells. In contrast, cells infected with Slo/Af at a MOI 1 showed a nearly 40-fold activation of the ISRE. Interestingly, cells infected with DOBV-afa showed a similar low activation as SK/Aa, while cells infected with DOBV-faf showed nearly the same activation (40-fold) as Slo/Af. Generally, a MOI-dependent induction pattern could be examined; with decreasing MOI, the activation signals declined, too. These results indicate that the different activation of ISRE is influenced by the S and L segment of the DOBV genome. The exchange of the M segment did not alter the activation of the ISRE.

5. Discussion

In this study it was shown that genetic reassortment between the European Dobrava-Belgrade hantavirus lineages SK/Aa and Slo/Af can be simulated in cell culture. These data confirm previous suggestions that reassortment processes could happen in nature and contribute to the evolution within the DOBV species (Klempa et al., 2003). Very recently, Schlegel et al. (2009) have found massive spill-over events of the *A. agrarius*-borne DOBV-Aa to *A. flavicollis* animals. The co-infection of the same animal (e.g., *A. flavicollis*) by DOBV-Aa and DOBV-Af is the precondition for their genetic reassortment in nature.

5.1 Virological and molecular tools for investigation of genetic reassortment

For enabling a fast and efficient screening of several viral clones, a Multiplex PCR was established. With primers specific for the particular virus strain and segment, our Multiplex PCR approach allowed the detection of fragments of all three segments in one amplification tube (Table 9). Moreover, the combination of two strain-specific assays (one specific for SK/Aa and one specific for Slo/Af) and the amplification of fragments of the L segment of both virus strains minimise the probability of false reassortment detection through PCR contamination (Figure 7). In addition, this multiplex approach allows an easy distinction between all reassortment scenarios, non-reassorted parental viruses, and diploid viruses. Together with the recently developed focus purification assay (Rang et al., 2006, Figure 6) the MP-PCR proved to be a powerful combination which allowed to screen more than 200 single viral clones and thus to obtain reasonable estimates of reassortment frequencies between two closely related hantaviruses *in vitro*.

5.2 Generation of reassortants between two DOBV lineages in cell culture

To investigate the genetic reassortment between two related Dobrava-Belgrade virus lineages in cell culture, co-infection experiments using different MOIs were performed (4.2.1). For the co-infection experiments, an overall MOI of 0.5 was taken. Three different ratios of the parental viruses were used for the co-infection experiment, either 1:1 or 9:1 and 1:9 (Table 12).

In total, 207 viral clones were isolated using the recently described focus purification assay (Rang et al., 2006) and analysed by established multiplex PCR approach (4.1).

In earlier studies with HCPS-associated viruses Sin Nombre (SNV) and Black Creek Canal virus (BCCV), it was suggested that reassortment occurs in nature and can be simulated in cell culture (Henderson et al., 1995; Rodriguez et al., 1998).

Henderson et al. (1995) analysed rodent samples collected from several counties in Nevada and eastern California. Sequence analyses of the rodent samples demonstrated the coexistence of two distinct SNV lineages in various regions of Nevada and eastern California and furthermore indicated that genetic reassortment occurred in nature between SNV belonging to distinct lineages. Moreover, the data show that most of these reassortants representing the exchange of the M segment and, to a minor extent, of S or L segment exchanges. These findings correlate well with an earlier genetic analysis of two SNV isolates also suggesting that RNA segment reassortment occurred (Li et al., 1995).

Rodriguez et al. (1998) described the first investigation of genetic reassortment between two related SNV strains and more distantly related SNV and BCCV in cell culture. Among 294 progeny viruses after dual infection of VeroE6 cells with two SNV strains (strains NMR11 and CC107), 25 (8.5%) genetically reassorted virus progenies were detected by multiplex RT-PCR. In this study, reassortment of the S, M and L segments was detected. Furthermore, over 29% of the progeny viruses appeared to contain S or M segments originating from both parental strains. After further passaging in cell culture, these diploids lost their diploid genotype yielding either parental or reassortant genotypes. Interestingly, only the NMR11 parental genotype was detected, suggesting that NMR11 outcompeted CC107 in the co-infection of VeroE6 cells. In addition, co-infection with the more distantly related hantaviruses SNV and BCCV yielded one reassortant progeny out of 163 (0.6%) screened progeny plaques.

McElroy et al. (2004) investigated the genetic reassortment between SNV and ANDV. Out of 208 screened virus clones only four (1.9%) were found to be reassortants. Furthermore only one type of stable reassortant virus, comprising the S and L segments of SNV and the M segment of ANDV, was examined. Analogous to that finding is the investigation of Rizvanov et al. (2004). Co-infection of VeroE6 cells with SNV and ANDV yielded a monoploid reassorted genotype in 10 out of 337 isolated viral clones. The genotype of these reassortants contained the S and L segment of SNV and the M segment of ANDV. The majority of progeny viruses revealed the ANDV genotype (281/337) and only 26 the parental SNV genotype. Twenty of the isolated viral plaques

were identified as diploids and the majority of the diploid segments involved the S and M segments.

In comparison to analogous *in vitro* experiments with New world hantaviruses, the observed reassortment frequency for closely related DOBV strains in this study was substantially higher. From the 207 isolated viral clones, 65 (31%) were identified as reassortants. Moreover, high numbers (65/207 or 31%) of virus progenies were detected with a diploid genotype.

In this study, three different co-infection experiments were carried out. For each experiment, different ratios of parental viruses were used for the co-infection experiments (Table 12). In experiment A, an equal ratio of both parental viruses was used for the co-infection. One hundred and four clones were isolated from that experiment. Most of the isolated progenies were identified as reassortants (43%). Several clones (35%) were identified as diploids and to a lesser extent also progenies with the parental genotypes were detected (9% SK/Aa genotype and 13% Slo/Af genotype). For the other two experiments, a ratio of 9:1 for the parental viruses was used for infection. In experiment B, where the concentration of SK/Aa was nine-fold higher then Slo/Af, the majority of the isolated viral progenies were identified as parental SK/Aa. In experiment C, where Slo/Af was used in a nine-fold higher concentration, the majority of virus progenies were detected as parental Slo/Af (Table 13). These results suggest that the respective parental virus available in a higher concentration outcompetes the virus in the lower concentration.

5.3 Two reassortment patterns detected

Although a high number of reassortants (n=65) was detected; only two out of six possible reassortment scenarios could be detected (4.2.3). In both cases, S and L segments of the same parental virus were combined with the heterologous M segment (afa and faf genotypes) (Figure 9).

Interestingly, in reassortment studies with New World hantaviruses analogous patterns were observed. Reassortants between SNV and ANDV (McElroy et al., 2004; Rizvanov et al., 2004) yielded only one stable reassortment pattern where the S and L segments of SNV and the M segment of ANDV were present in the virion. Also in reassortment studies with SNV and BCCV, only the exchange of the M segment was observed (Rodriguez et al., 1998). Reassortment combining S and L segments of different origin were found only for distinct strains of SNV species (Rodriguez et al., 1998).

Moreover, genetic reassortment was detected also in Puumala virus strains found in voles trapped in central Finland. Three out of the six possible reassortment combinations were detected between two closely related strains (Razzauti et al., 2008). Razzauti et al. (2009) showed also an inter-lineage genome segment reassortment. In this case, in agreement with our study, only two reassortment types were detected, where the S and L segments originated from the same genetic lineage (Razzauti et al., 2009).

These studies support our findings that the genetic reassortment in hantaviruses is not a random process. Only two stable reassortment patterns with homologous S and L segments were detected between two genetically distinct hantavirus lineages or even species. On the other hand, other segment combinations seem to be possible only between closely related strains within the same virus lineage.

It is possible that a specific interaction between the S and L segments or their products (N and L proteins) is necessary during the virus replication. Probably, this specificity is based on RNA-protein interactions (of the viral polymerase with the S segment RNA molecule) or protein-protein interactions (of the N and L proteins). However, these two hypotheses need to be further evaluated.

5.4 Diploids

As in the case of previous studies (Rizvanov et al., 2004; Rodriguez et al., 1998), a high proportion of the analysed progeny virus clones was found to have a diploid character. They contained both parental versions of at least one genome segment. Moreover, the most frequent diploid pattern found in this study involved S and L segments of the same virus and both versions of the M segment, too. In one viral clone, all segments of both parental viruses were detected.

Nine diploids were further analysed and treated. After three additional steps of passaging and focus purification, all of them lost their diploid character yielding in most cases parental SK/Aa genotypes and just in one case the reassortant faf genotype (Table 15).

5.5 Confirmation of genetic reassortment by sequence and molecular phylogenetic analysis of the complete S, M segments and L-ORF

To confirm the genetic reassortment and to examine whether particular mutations accumulated during the reassortment process, nucleotide sequence analyses were performed.

The complete nucleotide sequences of the S and M genomic segments and complete L segment-ORF for five reassortants (for afa genotype: A6, B-13 and for faf genotype: A35, A36 and B5) as well as parental viruses were obtained. For parental SK/Aa, the sequences of the complete S and M genomic segments were recently obtained (Klempa et al., 2004). For the Slo/Af strain used in this study S and M segments were also re-sequenced since the sequences available in GenBank were released in 1994 and could differ from the sequences of the current virus stock. The L segment-ORF of SK/Aa and Slo/Af were completely sequenced since only partial sequences of the L segments are available in GenBank.

Comparison of the complete sequences of the reassortants with the sequences of SK/Aa available in GenBank and the re-sequenced Slo/Af made it possible to establish a list of mutations which might be associated with the reassortment process. In general, the number of mutations was very low and no clear reassortment-associated pattern could be found (Table 16 and Table 17). Interestingly, a 24 nt deletion in the S segment 3'NCR was found in one of the analysed virus clones (A36), which suggests that this region is not essential for replication. The highest number of mutations was found in the L segment but this phenomenon is most likely connected with the large segment length and should not have functional relevance. Nevertheless, the list of amino acid exchanges provided could be a useful starting point for reassortment analyses in a future reverse genetics system. The detected mutations could be artificially introduced to the recombinant virus polymerase and its efficiency to use templates derived from different virus strains/species could be evaluated.

Moreover, mixed infection of a host cell is a basic prerequisite for reassortment, but it can also lead to intragenic homologous recombination. It is generally assumed that the negative-sense RNA viruses do not recombine because their RNA genome is tightly packaged into nucleocapsids. However, few exceptions were reported particularly for hantaviruses. In studies with wild-type Tula virus (TULV), it was shown that intragenic recombination appeared during the evolution of a TULV lineage in Slovakia

(Sibold et al., 1999a). Furthermore, homologous recombination events were observed also in transfection-mediated recombination experiments with TULV in cell culture (Plyusnin et al., 2002). The role of recombination in hantavirus evolution needs to be further evaluated. However, it seems that this mechanism plays a minor role compared to the classical genetic drift (accumulation of point mutations and deletions/insertions) or the reassortment of genomic segments.

Since the co-infection of the same cell is the prerequisite for both intragenic recombination and homologous reassortment, recombination analysis was performed too. Moreover, similarity plots were prepared to exclude the possibility that the observed pattern in the MP-PCR was not a result of homologous recombination instead of assumed reassortment. Obtained similarity plots gave no evidence for homologous recombination in the analysed reassortants clones and thereby confirmed their reassorted character (Figure 11).

5.6 Parental viruses differentially induced innate antiviral response

It is important to note that infection by DOBV-Af in south-east Europe causes a higher percentage of severe and lethal clinical cases than it was observed for infections by DOBV-Aa in Central and East Europe (Dzagurova et al., 2009; Kruger and Klempa, 2010). For other hantaviruses it was shown before that different virulence in humans is paralleled by corresponding in vitro findings, in the same way as differential induction of innate immune responses in host cells (Alff et al., 2006; Alff et al., 2008; Geimonen et al., 2002; Handke et al., 2009; Kraus et al., 2004; Schonrich et al., 2008; Spiropoulou et al., 2007). Prospect Hill and Tula hantaviruses considered to be non-pathogenic or of low virulence in humans, respectively, are known to induce early and pronounced innate antiviral responses. However, the pathogenic hantaviruses Hantaan virus (HTNV), SNV, ANDV, and New York virus (NYV), induce these responses in a retarded manner. The mechanisms and determinants underlying these differential responses of pathogenic and (rather) non-pathogenic hantaviruses are not resolved.

5.6.1 Influence of RIG-I for hantavirus replication

RIG-I stands at the beginning of a cascade that involves several transcription factors merging in the expression of type-I IFN. To assess the influence of RIG-I on hantavirus replication, the growth of SK/Aa, Slo/Af and their reassortant progenies in A549 cells

lacking RIG-I expression in comparison to the respective wild-type cell line was examined.

In general, the parental viruses as well as the reassortants could replicate in the wild-type cell line also in the RIG-I knock down cell line (ΔRIG-I) (Figure 13). Interestingly, parental SK/Aa and the afa reassortant could grow more efficiently in RIG-I knock down than in wild type A549 cells. This means that RIG-I sensing inhibits the replication of the less virulent SK/Aa but not of the virulent Slo/Af. For the growth of parental Slo/Af and the faf reassortants, the absence of RIG-I seems to have no effect. Thus, parental SK/Aa and the afa reassortants on one hand and Slo/Af and faf reassortants on the other hand can also be grouped together with regard to their growth characteristics in different cell lines.

These results may suggest that RIG-I has a suppressing influence of the replication of parental SK/Aa virus. Furthermore, they indicate that the exchange of the M segment has no influence on the characteristic RIG-I-associated replication pattern. In addition, it should be mentioned that A36 (faf genotype reassortant) was used for the growth kinetics. This reassortant has a deletion of 24 nucleotides in the 3´-non-coding region. Whether this deletion has an influence of the replication or other physiological characteristics remained unclear and needs to be further analysed.

5.6.2 Activation of the ISRE and MxA

Recently it was shown that pathogenic and non-pathogenic hantaviruses differentially induce the innate immune response in the established cell lines A549 and HuH7 (Handke et al., 2009). Based on this established cell system, the activation of the IFN-stimulated response element (ISRE) and the expression of the IFN-inducible MxA protein were examined as markers to monitor hantavirus-induced activation of the IFN system. It was shown that in A549 cells, the pathogenic SK/Aa and Slo/Af trigger retarded and non-detectable, respectively, innate responses are similar to those of the phenotype observed in HTNV infected cells. It would be interesting to test whether TLR3 is required for the activation of this retarded response as shown for HTNV in this experimental system (Handke et al., 2009).

For investigating the activation of the ISRE reporter gene, HuH7 cells were transfected using a luciferase expression construct controlled by an IFN-stimulated response element and infected with SK/Aa, Slo/Af and their reassortant progenies afa and faf. One day post infection the cells were harvested to quantify the luciferase activity. In this

experiment it could be shown that SK/Aa and Slo/Af differentially induce the ISRE. In comparison to uninfected cells SK/Aa activated the transfected ISRE five-fold higher, whereas Slo/Af showed a nearly 40-fold activation (Figure 15). Interestingly, both reassortants show ISRE activation patterns according to the origin of their S and L genomic segments; reassortant afa resembled SK/Aa in its ISRE activation pattern and reassortant faf resembled parental Slo/Af.

Furthermore, Western blot analyses for the expression of the antiviral MxA protein also revealed two distinct activation patterns. In A549 cells infected with SK/Aa, retarded activation of MxA on day four after infection was detected, but no activation in HuH7 infected cells. In contrast to the effect of SK/Aa, no Slo/Af-induced expression of MxA protein in A549 cells was detected, but an early and strong MxA expression in HuH7 cells was observed, which decreased steadily with the establishment of the infection (Figure 12).

This result might suggest that at later time points during infection, Slo/Af develops an antagonistic activity which interferes with the activation of the IFN response or directly targets MxA for degradation. Whether DOBV Slo/Af can increase the turnover of MxA protein as recently shown for the double stranded RNA-dependent protein kinase (PKR) by Rift Valley Fever virus (Habjan et al., 2009) has to be further investigated.

As in the case of ISRE induction, the two reassortants followed the MxA induction pattern of the parental viruses (Figure 12). According to this, the afa reassortant followed the MxA induction pattern of SK/Aa whereas the faf reassortant resembled the Slo/Af phenotype. Moreover, the results of the experiments for the activation of the ISRE and the expression of the MxA protein indicate that the exchange of the M segment did not effect the virus-specific activation of the innate immune response. This suggests S and L segments as genetic determinants for virus type-specific induction/modulation of innate immune responses by hantaviruses.

For the pathogenic NYV, it was shown that the (M-segment encoded) glycoprotein G1 cytoplasmic tail can directly block TRAF3-TBK1 complex formation and TBK1- and RIG-I-directed IFN responses (Alff et al., 2006; Alff et al., 2008). These findings are contrary to our data. In our study it was shown that the glycoprotein (and therewith its cytoplasmic tail) is not crucial for the differential responses observed in SK/Aa- and Slo/Af-infected HuH7 cells, because the same early transient MxA expression as by Slo/Af was also induced by the faf reassortant, which carried the M segment from

SK/Aa. Thus, the S and L segments should contain the determinants decisive for activation or inhibition of the described differential responses.

Recently it was reported that ectopic expression of hantavirus nucleocapsid proteins can inhibit TNF-α-induced activation of NF-κB. Furthermore, HTNV, Seoul virus, and DOBV nucleocapsid proteins were shown to interact with different importin-α subunits involved in shuttling of cargoes including NF-κB from the cytoplasm into the nucleus (Taylor et al., 2009b; Taylor et al., 2009a). In the context of our study it would be interesting to test whether nucleocapsids of SK/Aa and Slo/Af might differentially interact with importin-α. Furthermore, it would be interesting to see whether this interaction also affects not only inflammatory but also antiviral responses including activation of ISRE and expression of type I IFN.

The pathogenic DOBV SK/Aa induced only a minor and retarded innate response as also shown for HTNV in vitro. In the infected human organism, this limited response might lead to increased dissemination and represent an important determinant for pathogenicity. Based on the presented results, we assume that compared to DOBV SK/Aa, DOBV Slo/Af should require higher infectious doses to overcome early induction of antiviral responses and to establish an infection. Thereafter, however, DOBV Slo/Af might lead to a more severe course of disease due to the proposed antagonistic activity which blocks innate immunity.

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APPENDIX

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Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version nicht veröffentlicht.

List of own Publications

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Kirsanovs, S., Klempa, B., Krüger, D.H.: In vitro reassortment between Dobrava virus lineages. Annual Meeting of the Society of Virology (GfV), Heidelberg, Germany, March 2008 – poster

Declaration

Herewith I declare that I prepared the present thesis with the title: “Genetic reassortment between members of different Dobrava-Belgrade virus lineages and allocation of innate immune response modulation to particular genome segments” myself and did not make use of any other utilities and literature as they are mentioned in the thesis. All citations are marked and listed in the references.
I also do not presented the thesis to any other audit committee.

Date	Signature
07.06.2010	Sina Kirsanovs

Erklärung

Ich, Sina Kirsanovs, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: “Genetic reassortment between members of different Dobrava-Belgrade virus lineages and allocation of innate immune response modulation to particular genome segments” selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.

Datum	Unterschrift
07.06.2010	Sina Kirsanovs