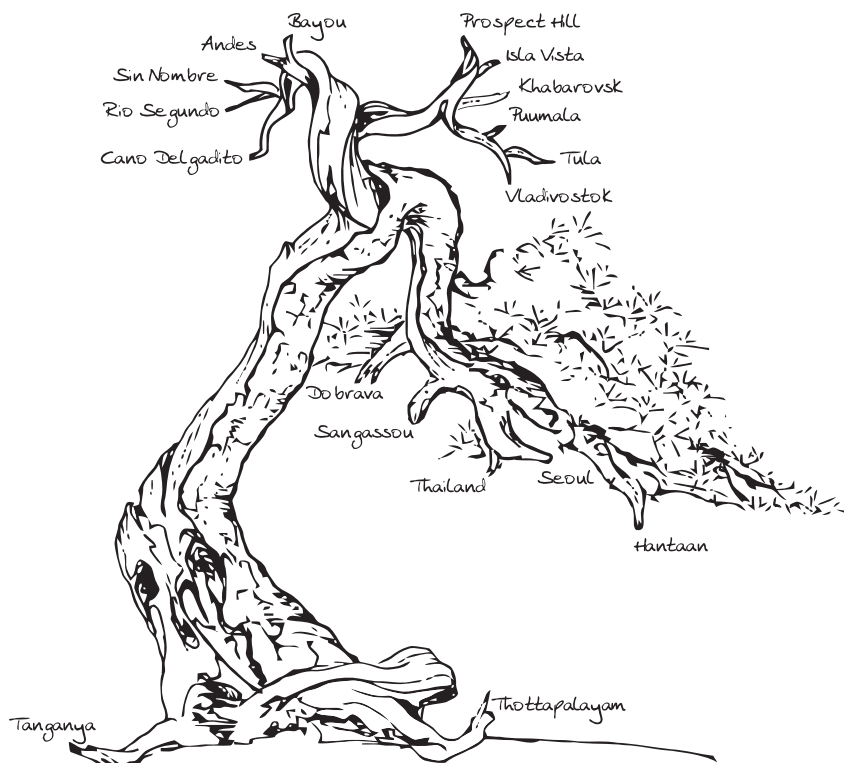




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# HANTAVIRUSES: CLASSIFICATION, DIAGNOSIS AND PREVENTION



**PIET MAES**

Promotor: Prof. Dr. Marc Van Ranst

Proefschrift voorgedragen tot het behalen van de graad van Doctor in de Medische Wetenschappen  
Leuven, 2007





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**Piet Maes**

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*Nature shows us only the tail of the lion. But I have no doubt that the lion belongs with it, even if he cannot reveal himself all at once. We see him only the way a louse that sits upon him would.*

Albert Einstein

## DANKWOORD

---

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A handwritten signature in black ink, consisting of a large, stylized 'R' followed by the letters 'iet' in a cursive script.

# TABLE OF CONTENTS

SUMMARY.....	1
SAMENVATTING .....	5

## CHAPTER 1

### GENERAL INTRODUCTION

1. THE HANTAVIRUSES .....	10
1.1 <i>Introduction</i> .....	10
1.2 <i>Historical background</i> .....	10
1.3 <i>Genome organization</i> .....	12
1.4 <i>Hantavirus taxonomy and classification</i> .....	13
1.5 <i>Epidemiology of hantavirus infections</i> .....	16
2. HUMAN HANTAVIRUS DISEASE AND PATHOGENESIS .....	16
3. LABORATORY DIAGNOSIS OF HANTAVIRUS INFECTIONS .....	18
4. PREVENTION OF HANTAVIRUS INFECTIONS .....	19
4.1 <i>Vaccine strategies against hantaviruses</i> .....	19
4.2 <i>Antiviral therapy</i> .....	20
4.3 <i>Rodent control</i> .....	22
5. AIMS AND OBJECTIVES .....	22

## CHAPTER 2

### HANTAVIRUS GENETIC CLASSIFICATION BASED ON NUCLEOCAPSID PROTEIN SEQUENCES

1. SUMMARY .....	26
2. INTRODUCTION.....	26
3. MATERIALS AND METHODS .....	28
3.1 <i>Hantavirus sequences</i> .....	28
3.2 <i>Similarity calculation</i> .....	29
3.3 <i>Neighbor-joining and maximum likelihood analysis</i> .....	29
3.4 <i>Bootscreening analysis</i> .....	29
4. RESULTS.....	31
4.1 <i>Similarity distribution of hantaviruses</i> .....	31

4.2	<i>Definition of hantavirus groups</i>	33
4.3	<i>Definition of hantavirus species</i>	33
4.4	<i>Definition of hantavirus strains</i>	34
4.5	<i>Determination of the minimum sequence length</i>	36
4.6	<i>Comparison with the ICTV defined hantavirus species</i>	38
4.7	<i>Multiple alignment of the hantavirus sequences</i>	38
4.8	<i>Similarity and identity calculation methods</i>	39
5.	DISCUSSION	39
5.1	<i>Sequence-based classification of hantaviruses</i>	39
5.2	<i>'Problematic' hantavirus species</i>	40
5.2.1	Topografov virus	40
5.2.2	New York virus	40
5.2.3	South American hantaviruses	41
5.3	<i>Tentative novel hantavirus species</i>	42
5.3.1	Sangassou virus	42
5.3.2	Tanganya virus	42
5.3.3	Vladivostok virus	42
5.4	<i>Conclusions</i>	42
6.	REFERENCES	52

### CHAPTER 3

#### GETHA: A WEB-BASED GENOTYPING TOOL FOR HANTAVIRUSES

1.	SUMMARY	56
2.	INTRODUCTION	56
3.	IMPLEMENTATION	57
3.1	<i>S segment reference sequences</i>	57
3.2	<i>Web application procedure</i>	58
4.	RESULTS AND DISCUSSION	59
5.	REFERENCES	60

### CHAPTER 4

#### DETECTION OF PUUMALA VIRUS ANTIBODY WITH ELISA USING A RECOMBINANT TRUNCATED NUCLEOCAPSID PROTEIN EXPRESSED IN *ESCHERICHIA COLI*

1.	SUMMARY	64
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2. INTRODUCTION.....	64
3. MATERIALS AND METHODS .....	65
3.1 <i>Construction of the expression plasmid</i> .....	65
3.2 <i>Expression and purification of the recombinant protein</i> .....	65
3.3 <i>Western blotting</i> .....	66
3.4 <i>Serum samples</i> .....	66
3.5 <i>Enzyme-linked immunosorbent assay (ELISA)</i> .....	66
4. RESULTS.....	67
4.1 <i>Recombinant protein</i> .....	67
4.2 <i>Puu118 ELISA</i> .....	68
5. DISCUSSION.....	70
6. REFERENCES .....	72

## CHAPTER 5

### REPLICATION REDUCTION NEUTRALIZATION TEST, A QUANTITATIVE RT-PCR-BASED TECHNIQUE FOR THE DETECTION OF NEUTRALIZING HANTAVIRUS ANTIBODIES

1. SUMMARY .....	76
2. INTRODUCTION.....	76
3. MATERIALS AND METHODS .....	77
3.1 <i>Virus and cell culture</i> .....	77
3.2 <i>Serum samples</i> .....	78
3.3 <i>Primer and fluorogenic probe design</i> .....	78
3.4 <i>Hantavirus quantitation</i> .....	78
3.5 <i>Quantitative RT-PCR (qRT-PCR)</i> .....	79
3.6 <i>Replication reduction neutralization test (RRNT)</i> .....	80
3.7 <i>Focus reduction neutralization test (FRNT)</i> .....	80
3.8 <i>Statistic analysis</i> .....	80
4. RESULTS.....	81
4.1 <i>Standardization and quantitation of qRT-PCR</i> .....	81
4.2 <i>Optimization and application of qRT-PCR to virus neutralization test</i> .....	81
4.3 <i>Neutralization test using qRT-PCR with mouse sera</i> .....	84
4.4 <i>Neutralization test using qRT-PCR with HFRS patient sera</i> .....	84
5. DISCUSSION.....	86
6. REFERENCES .....	88

## CHAPTER 6

### TRUNCATED RECOMBINANT DOBRAVA VIRUS NUCLEOCAPSID PROTEINS, INDUCE STRONG, PROTECTIVE AND LONG-LASTING IMMUNE RESPONSES IN MICE

1. SUMMARY .....	92
2. INTRODUCTION.....	92
3. MATERIALS AND METHODS .....	93
3.1 <i>Construction, expression and purification of the recombinant proteins</i> .....	93
3.2 <i>Western blotting</i> .....	94
3.3 <i>Animal immunizations</i> .....	94
3.4 <i>DOBV-challenge experiments</i> .....	95
3.5 <i>Detection of Np-specific antibodies</i> .....	95
3.6 <i>Replication reduction neutralization test (RRNT)</i> .....	95
3.7 <i>Antigen-specific T cell detection by FACS</i> .....	96
4. RESULTS.....	97
4.1 <i>Expression and antigenicity of the DOBV rNp proteins</i> .....	97
4.2 <i>Humoral immune responses to DOBV rNp proteins</i> .....	98
4.3 <i>Protection of NMRI mice from infection with DOBV rNp proteins</i> .....	101
4.4 <i>Antigen-specific T-cell responses to DOBV rNp proteins</i> .....	102
5. DISCUSSION.....	103
6. REFERENCES .....	104

## CHAPTER 7

### TRUNCATED RECOMBINANT PUUMALA VIRUS NUCLEOCAPSID PROTEINS PROTECT MICE AGAINST CHALLENGE *IN VIVO*

1. SUMMARY .....	110
2. INTRODUCTION.....	110
3. MATERIALS AND METHODS .....	112
3.1 <i>Virus and cell culture</i> .....	112
3.2 <i>Construction, expression and purification of the recombinant proteins</i> .....	113
3.3 <i>Animal immunizations</i> .....	113
3.4 <i>PUUV-challenge experiments</i> .....	114
3.5 <i>Detection of Np-specific antibodies</i> .....	114
3.6 <i>Replication reduction neutralization test (RRNT)</i> .....	115

3.7 <i>Antigen-specific T cell detection by FACS</i> .....	116
4. RESULTS.....	116
4.1 <i>Expression and antigenicity of the PUUV rNp proteins</i> .....	116
4.2 <i>Humoral immune responses the PUUV rNp proteins</i> .....	117
4.3 <i>Protective efficacy of the PUUV rNp proteins</i> .....	119
4.4 <i>Antigen-specific T-cell responses to the PUUV rNp proteins</i> .....	120
5. DISCUSSION.....	121
6. REFERENCES .....	123

## CHAPTER 8

### CHLOROQUINE, AN ANTI-MALARIA DRUG AS PREVENTION FOR HANTAVIRUS INFECTIONS

1. SUMMARY .....	130
2. INTRODUCTION.....	130
3. MATERIALS AND METHODS .....	131
3.1 <i>Virus and cell culture</i> .....	131
3.2 <i>Compounds</i> .....	131
3.3 <i>Primer and fluorogenic probe design</i> .....	132
3.4 <i>Hantavirus quantitation by using quantitative RT-PCR (qRT-PCR)</i> .....	132
3.5 <i>Antiviral assay</i> .....	133
3.6 <i>Cytotoxic assay</i> .....	133
3.7 <i>In vivo evaluation of chloroquine in 1-day-old C57Bl/6 pups</i> .....	133
4. RESULTS AND DISCUSSION .....	134
5. REFERENCES .....	138

## CHAPTER 9

### GENERAL DISCUSSION AND REFLECTIONS

1. HANTAVIRUS NUCLEOCAPSID PROTEIN-BASED VACCINE .....	142
2. ANTIVIRAL THERAPY FOR HANTAVIRUS INFECTIONS .....	143
3. CLASSIFICATION OF HANTAVIRUSES.....	146
4. CONCLUDING REMARKS .....	147

REFERENCES .....	150
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PUBLICATION LIST .....	161
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## ABBREVIATIONS

<b>ANDV</b>	Andes virus
<b>APC</b>	allophycocyanin
<b>BAYV</b>	Bayou virus
<b>BCCV</b>	Black Creek Canal virus
<b>BSL</b>	biosafety level
<b>CADV</b>	Caño Delgadito virus
<b>CC<sub>50</sub></b>	50% cytotoxic concentration
<b>CMV</b>	cytomegalovirus
<b>CTL</b>	cytotoxic T lymphocyte
<b>DOBV</b>	Dobrava-Belgrade virus
<b>ELISA</b>	enzyme-linked immunosorbent assay
<b>ELMCV</b>	El Moro Canyon virus
<b>FACS</b>	fluorescent-activated cell sorting
<b>FITC</b>	fluorescein isothiocyanate
<b>FRNT</b>	focus reduction neutralization test
<b>HFRS</b>	hemorrhagic fever with renal syndrome
<b>HPS</b>	hantavirus cardiopulmonary syndrome
<b>HTNV</b>	Hantaan virus
<b>HUVEC</b>	human umbilical vein endothelial cells
<b>IAA</b>	3- $\beta$ -indoleacrylic
<b>IC<sub>50</sub></b>	50% inhibitory concentration
<b>ICONE</b>	improved cell for over and non-leaky expression
<b>ICTV</b>	International Committee on Taxonomy of Viruses
<b>IPTG</b>	isopropyl $\beta$ -D-thiogalactopyranoside
<b>ISLAV</b>	Isla Vista virus
<b>KHAV</b>	Khabarovsk virus
<b>LANV</b>	Laguna Negra virus
<b>MULV</b>	Muleshoe virus
<b>NCR</b>	non-coding region
<b>NE</b>	nephropathia epidemica
<b>NMRI</b>	Naval Medical Research Institute

<b>Np</b>	nucleocapsid protein
<b>NYV</b>	New York virus
<b>OD</b>	optical density
<b>PBMC</b>	peripheral blood mononuclear cell
<b>PCR</b>	polymerase chain reaction
<b>PE</b>	phycoerythrin
<b>PerCP</b>	peridinin chlorophyll protein
<b>PHV</b>	Prospect Hill virus
<b>PID</b>	percentage identity
<b>PUUV</b>	Puumala virus
<b>PVDF</b>	polyvinylidene difluoride
<b>qRT-PCR</b>	quantitative reverse-transcriptase polymerase chain reaction
<b>RIOMV</b>	Rio Mamore virus
<b>RIOSV</b>	Rio Segundo virus
<b>RRNT</b>	replication reduction neutralization test
<b>RT-PCR</b>	reverse-transcriptase polymerase chain reaction
<b>SEB</b>	Staphylococcus enterotoxin B
<b>SEOV</b>	Seoul virus
<b>SNV</b>	Sin Nombre virus
<b>TCID</b>	tissue culture infectious dose
<b>THAIV</b>	Thailand virus
<b>TMPV</b>	Thottapalayam virus
<b>TOPV</b>	Topografov virus
<b>TULV</b>	Tula virus
<b>VLP</b>	virus-like particle
<b>WHO</b>	World Health Organization

## SUMMARY

Hantaviruses are rodent-borne RNA viruses within the family Bunyaviridae. They are found worldwide and are associated with two severe disease syndromes: hemorrhagic fever with renal syndrome in Asia and Europe, and hantavirus pulmonary syndrome in the Americas. Human infection is usually initiated by inhalation of aerosols of excreta from infected rodents and can result in high mortality rates. These viruses are currently divided into 22 distinct species based on four rules defined by *The International Committee on Taxonomy of Viruses* or ICTV. A correct classification of hantavirus is an essential tool for antiviral therapy and vaccine development since researchers and medical doctors need to be able to identify for example the correct hantavirus species circulating in a specific geographic region. Unfortunately, the current classification as defined by the ICTV is rather difficult to comply and therefore often ignored by the descriptors of novel hantavirus strains. In order to obtain a scientifically based genetic classification in addition to the current ICTV classification, we analyzed in a first part of this thesis, complete S, M and L segment sequences found in Genbank by using similarity comparison, together with maximum likelihood and neighbor-joining analysis. S segment amino acid sequence comparison allowed a clear distinction between the different hantavirus species and lead us to propose an adjustment of the second rule of the ICTV classification guidelines (“*a hantavirus species exhibits an at least 7% difference in amino acid identity when comparing the complete S segment and M segment sequences*”) to a more appropriate rule, “*a 10% difference in S segment similarity or a 12% difference in M segment similarity based on complete amino acid sequences*“ in accordance with the current situation in the hantavirus field. Furthermore, the proposed guideline can replace the neutralization tests, to genetically identify them. For hantaviruses discovered without a virus isolate, these guidelines can form an alternative, scientifically based approach in identifying the correct hantavirus species. In order to draw clear and distinct dividing lines between species of hantaviruses, we developed an automated web-based tool which uses amino acid sequences from the S segment. This online GeTHa (Genotyping Tool for Hantaviruses) application was developed using Java and PERL scripts and combines phylogenetic analyses with similarity comparison for the genetic classification of hantaviruses. The analyses of previously well-characterized hantavirus sequences found in Genbank showed that all tested sequences could be correctly classified when using the GeTHa web application.

In Europe, the Dobrava-Belgrade virus and Puumala virus are the major hantaviruses that cause hemorrhagic fever with renal syndrome in humans. As hantaviruses can cause diseases with high morbidity and mortality, and as to date there is no specific treatment, research efforts are concentrated on the development of vaccines and antiviral therapies. In a second part of this doctoral thesis, we characterized the immunogenicity of recombinant nucleocapsid proteins of the Dobrava-Belgrade virus and Puumala virus, some of them linked to a carrier protein corresponding to the outer membrane protein A from *Klebsiella pneumoniae* (rP40). This rP40 molecule is a novel carrier protein which facilitates exogenous antigen uptake by dendritic cells. We cloned and expressed several recombinant Dobrava-Belgrade virus and Puumala virus proteins in the *E. coli* mutant ICONE 200 using the tryptophan promoter controlled pTEXmp18 expression vector. To test these recombinant proteins for their immunogenicity in NMRI mice, we developed an ELISA based on the Dob118 and Puu118 recombinant proteins, two proteins consisting of the first 118 amino-terminal amino acids of respectively the Dobrava-Belgrade virus and Puumala virus S segment. Using this ELISA, all recombinant proteins were found to be highly immunogenic after 3 immunizations with 10 µg of the different recombinant proteins. The immunizations resulted in the induction of a strong nucleocapsid-specific IgG response with a predominance of IgG1 over IgG2b and IgG2a. A specific IgG3 response could not be detected. NMRI mice immunized with recombinant proteins without rP40 showed lower nucleocapsid-specific antibody responses in comparison with the rP40 conjugated constructs.

Although several species of rodents, rabbits and non-human primates can be infected with the Dobrava-Belgrade virus or Puumala virus, only humans have been shown to present symptoms of hemorrhagic fever with renal syndrome. We have used an NMRI mouse model to test the capacity of our recombinant proteins to induce protection against virus challenge. Infection in this NMRI model is scored by the presence of neutralizing antibodies. In our experiments, neutralizing antibodies were detected by using a new technique called the replication reduction neutralization test (RRNT). With this RRNT, the degree of reduction in virus replication after incubation with sera containing specific neutralizing antibodies is determined by means of quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). The detection of hantavirus genomes with qRT-PCR eliminates the need for animal anti-hantavirus antibodies to detect the residual hantavirus genomes as needed for other neutralization protocols. In the challenge experiments with NMRI mice, only the truncated constructs containing the first 118 N-terminal amino acids conjugated to rP40 (P40-Dob118, P40p-Dob118 and P40-Puu118) gave 100% protection after 3 immunizations with 10 µg

protein. These results suggest that these recombinant proteins are good candidates for a recombinant subunit vaccine against the Dobrava-Belgrade virus and the Puumala virus.

In a last part of this dissertation, we examined chloroquine, a 4-aminoquinoline, as an effective inhibitor of the replication of hantavirus *in vitro* and *in vivo*. Chloroquine is a clinically approved drug which is effective against malaria, and was tested *in vitro* with several New World and Old World hantavirus strains in Vero E6 cell culture. Results indicate that the IC<sub>50</sub> of chloroquine for antiviral activity ( $10.2 \pm 1.43 \mu\text{M}$ ) was significantly lower than its cytostatic activity, CC<sub>50</sub> ( $260 \pm 2.52 \mu\text{M}$ ), yielding a selectivity index of 25.5. In the *in vivo* experiments with newborn C57Bl/6 pups, The highest survival rate (72.7%) was seen with a concentration of 10 mg/kg. Survival rates declined in a dose dependent manner, with 47.6% survival when treated with 5 mg/kg chloroquine and 4.2% survival when treated with 1 mg/kg chloroquine. Our results show that chloroquine can be highly effective against hantavirus infection in newborn mice and may be considered as a future drug against hantaviruses.



## SAMENVATTING

Hantavirussen zijn wereldwijd verspreid. Knaagdieren en spitsmuizen sturen deze enkelstrengige RNA-virussen de wereld in met twee heel ernstige aandoeningen in hun kielzog: hemorragische koorts met niersyndroom (vooral in Azië en Europa) en het hantavirus longsyndroom (vooral in Noord- en Zuid-Amerika). De infectie wordt bij de mens veroorzaakt door contact met geïnfecteerde knaagdieren of hun uitwerpselen. De geïnfecteerde dieren zijn zelf geen slachtoffers van het virus maar ze kunnen wel een erg dodelijk spoor achterlaten. Men kent vandaag 22 soorten hantavirussen. Deze classificatie gebeurt op basis van vier basiscriteria vastgelegd door het ICTV (*International Committee on Taxonomy of Viruses*). Dergelijke classificatie is een essentieel hulpmiddel bij de ontwikkeling van vaccins en antivirale therapieën. Onderzoekers moeten voor hun regio immers precies weten met welk soort virus zij te kampen hebben om het juiste vaccin en de juiste therapie te kunnen ontwikkelen. In concrete situaties is de ICTV-classificatie helaas moeilijk toe te passen. Ze wordt dan ook zelden gebruikt bij de identificatie van nieuwe soorten hantavirussen. Precies daarom werd, in een eerste deel van deze studie, gezocht naar een nieuwe, op similariteit gebaseerde classificatie naast die van het ICTV. We analyseerden hiervoor alle bestaande en volledige sequenties van de hantavirus S-, M- en L-segmenten, meer bepaald via similariteits- en fylogenetische analyses (*maximum likelihood en neighbor-joining analyses*). Dankzij de hantavirus S-segment sequenties, kwamen wij tot een duidelijker onderscheid tussen de verschillende soorten hantavirussen. Wij stellen dan ook voor om de tweede regel van de ICTV-classificatierichtlijnen (een minimum verschil van 7% in aminozuuridentiteit in vergelijking met de volledige hantavirus S- en M-segmenten), aan de huidige situatie aan te passen. Die tweede regel wordt dan: “een minimum verschil van 10% in S-segment similariteit of een minimum verschil van 12% in M-segment similariteit”. Deze gewijzigde richtlijn kan bovendien ook worden gebruikt om hantavirussen voorlopig te classificeren tot de neutralisatietesten betere resultaten genereren. Voor hantavirussen die zonder isolaat worden ontdekt, waardoor geen neutralisatietesten kunnen worden uitgevoerd, kan deze wijziging een alternatief bieden voor een correcte identificatie. Om die identificatie nog te vereenvoudigen, hebben wij een webtoepassing ontwikkeld, gebaseerd op S-segment sequenties. Deze webtoepassing (met Java en Perl-scripts) combineert fylogenetische analyse met similariteitsvergelijking voor de classificatie van hantavirussen. Analyses met perfect gedefinieerde hantavirussequenties hebben overigens aangetoond dat onze webtoepassing deze sequenties perfect classificeert.

In Europa zijn vooral het Dobrava-Belgrade virus en het Puumala virus bij mensen oorzaak van hemorragische koorts met niersyndroom. Omdat hantavirussen infecties met hoge morbiditeit en mortaliteit veroorzaken, en gezien men hiervoor momenteel geen specifieke behandeling kent, zijn de onderzoeksinspanningen geconcentreerd op de ontwikkeling van vaccins en antivirale therapie. In een tweede deel van dit doctoraatsproject wordt de immunologische respons onderzocht in NMRI-muizen die behandeld werden met verschillende recombinante proteïnen, afgeleid van het S-segment nucleocapside van het Dobrava-Belgrade virus en het Puumala virus. Sommige van deze proteïnen bevatten het membraanproteïne A van *Klebsiella pneumoniae*, ook wel rP40 genoemd. Deze molecule vergemakkelijkt de opname van exogene antigenen door dendritische cellen en doet dienst als adjuvant. De verschillende Dobrava-Belgrade virus en Puumala virus recombinante proteïnen werden gekloneerd en tot expressie gebracht in de *E. coli* mutant ICON 200 via een tryptofaanpromotor-gecontroleerde pTEX-mp18 expressievector. Om de immunogeniciteit van deze recombinante proteïnen te testen, werd een ELISA ontwikkeld gebaseerd op Dob118 en Puu118, twee proteïnen bestaande uit de eerste 118 amino-terminale aminozuren van respectievelijk het Dobrava-Belgrade virus en het Puumala virus S-segment. Via deze ELISA's kon worden aangetoond dat alle geteste recombinante proteïnen een sterke immunologische reactie veroorzaakten na 3 vaccinaties met 10 µg proteïne. Deze vaccinaties resulteerden in de inductie van een sterke nucleocapside-specifieke IgG reactie met een overheersing van IgG1 over IgG2b en IgG2a isotype. Een specifieke IgG3 isotype reactie werd niet waargenomen. De NMRI-muizen gevaccineerd met de recombinante proteïnen zonder het rP40 proteïne, vertoonden lagere titers van nucleocapside-specifieke antilichamen in vergelijking met de proteïnen die wel rP40 bevatten.

Hoewel verscheidene soorten knaagdieren, konijnen en niet-humane primaten met het Dobrava-Belgrade virus of het Puumala virus geïnfecteerd kunnen worden, vertonen enkel mensen symptomen van hemorragische koorts met niersyndroom. Om het beschermende effect van de verschillende recombinante proteïnen na te gaan, werd gebruik gemaakt van een NMRI-muismodel. Infectie in dit NMRI-model wordt bevestigd door middel van de aanwezigheid van neutraliserende antilichamen. In onze experimenten werden neutraliserende antilichamen gedetecteerd dankzij een nieuwe techniek, de *replication reduction neutralization test* (RRNT). Met deze test wordt de reductiegraad van het virus na incubatie met serum bepaald door middel van een kwantitatieve RT-PCR (qRT-PCR). In de infectie-experimenten gaven enkel de proteïnen bestaande uit de eerste 118 N-terminale aminozuren gekoppeld aan rP40 (P40-Dob118, P40p-Dob118 en P40-Puu118) 100% bescherming en dit na 3 vaccinaties met 10 µg proteïne. Deze resultaten suggereren dat deze recombinante proteïnen goede kandidaten

zijn voor een recombinant subunit vaccin gericht tegen het Dobrava-Belgrade virus en het Puumala virus.

In het laatste deel van deze studie, bestuderen we chloroquine, een 4-aminoquinoline, als efficiënte inhibitor van de replicatie van het hantavirus *in vitro* en *in vivo*. Chloroquine, een klinisch goedgekeurd middel tegen malaria, werd *in vitro* getest in Vero E6 celcultuur met verschillende Nieuwe en Oude Wereld hantavirussen. De resultaten wijzen erop dat de IC<sub>50</sub> van chloroquine voor antivirale activiteit ( $10,2 \pm 1,43 \mu\text{M}$ ) beduidend lager is dan zijn cytotoxische activiteit, CC<sub>50</sub> ( $260 \pm 2,52 \mu\text{M}$ ), wat een selectiviteitsindex van 25,5 opbrengt. In de *in vivo* experimenten met pasgeboren C57Bl/6-muizen, werd na infectie de hoogste overleving waargenomen (72,7%) bij muizen dagelijks behandeld met 10 mg/kg chloroquine. De overleving van de pasgeboren C56Bl/6-muizen daalde op een dosisafhankelijke wijze, met 47,6% overleving bij een dagelijkse behandeling met 5 mg/kg chloroquine, met 4,2% overleving wanneer er dagelijks werd behandeld met 1 mg/kg. Onze resultaten tonen aan dat chloroquine heel efficiënt is tegen hantavirusinfectie bij pasgeboren C57Bl/6-muizen en als toekomstig preventief middel tegen hantavirussen kan worden beschouwd.



## GENERAL INTRODUCTION

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This chapter is based on the following publications:

**Piet Maes, Jan Clement, Irina Gavrillovskaya, and Marc Van Ranst.** 2004. Hantaviruses: Immunology, treatment and prevention. *Viral Immunology* 17(4), pp 481-497.

**Jan Clement, Piet Maes, and Marc Van Ranst.** 2006. Hantaviruses in the Old and New World. Pp 161-177 In: *Perspectives in Medical Virology: Emerging Viruses in Human Populations*, E. Tabor (editor). Elsevier, London, UK.

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## 1. THE HANTAVIRUSES

### 1.1 Introduction

The family Bunyaviridae is divided into five genera: *Bunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus*, and *Tospovirus*. All viruses in the family except the hantaviruses are arboviruses (arthropod-borne viruses), and are maintained in nature, usually in silent, sylvatic transmission cycles, between susceptible vertebrate hosts (or plant hosts for the tospoviruses) and hematophagous arthropods, such as mosquitoes, biting flies, ticks, or by thrips. Vertebrate hosts of the arboviruses include rodents and other small mammals, primates, birds, and ungulates. Tospoviruses infect more than 300 species of plants. Hantaviruses are not known to productively infect arthropods, but instead are maintained in nature in persistently-infected rodents and are transmitted in infectious aerosols of urine, feces, or saliva.

The genus *Hantavirus* comprehends 22 different hantavirus species. Hantavirus is the only hemorrhagic fever virus with an ubiquitous, worldwide distribution, including the temperate regions of the northern hemisphere. The majority of hantavirus species known until 1993 had the kidney as the primary target organ, this explains why the historically first denomination of the disease (in the western world) was “*nephropathia epidemica*” (NE). Nowadays, the most commonly used name for this affection is the official WHO denomination “*hemorrhagic fever with renal syndrome*” (HFRS). This term is now mainly used for describing Hantaan virus induced disease in Asia and Eastern Russia, where the clinical picture is often more severe than in the Puumala virus induced NE, prevalent in Europe. Sin Nombre virus however, isolated after a 1993 epidemic in the USA, seems to affect primarily the lungs, where it causes a viral form of adult respiratory distress syndrome, now called “*hantavirus cardiopulmonary syndrome*” or HPS (Duchin et al., 1994; Hjelle et al., 1995).

Although only recently recognized, hantaviruses are not to be considered “new” or even “emerging” viruses, since phylogenetic studies provided evidence to the fact that they are the product of millions of years of co-evolution with their respective rodent hosts. This explains the differences between hantaviruses of the New and the Old World, but also some common features.

### 1.2 Historical background

Human conflicts during the last centuries played a prominent role in elucidating the etiology, clinical evolution, epidemiology and ecology of hantavirus diseases. During the American Civil War (1862-1863) 14,000 of such disease cases were described among the Northern Armies of the Central Region (Clement and van der, 1987). On the other hand during the

South African Boer War, the Japanese-Russian War, the Spanish-American War or the French-German War in 1870, no mention is found of a similar epidemic (Clement and van der, 1987), proving at least that the responsible agent at those periods was not ubiquitous. The term “*Field nephritis*” or “*Kriegsnephritis*” was coined for the first time in World War I, where from 1915 on, several thousand cases were reported among both allied and German troops in the trenches of Flanders’ battlefields (Bradford J.R., 1916; Clement and van der, 1987). An annual variability and an almost exclusive occurrence in the trenches was noted. This disease may well have been caused by a hantavirus now known to occur in western Europe and Scandinavia.

When Japanese troops invaded Manchuria in the mid 1930s, they were confronted with the so-called Songo fever and suffered 12,600 cases. Japanese military doctors already suspected and later in 1942 proved a viral etiology (Ishii S. et al., 1942). On the European front, 16,000 cases of an epidemic clinically similar to the now well-characterized disease NE, were noted among German troops in Finnish Lapland and in Yugoslavia (Stuhlfauth K., 2007; Hortling H., 1944).

During yet another armed conflict in Asia, the Korean War (1951-1953), Western medicine was suddenly confronted for the first time with an up to then unknown acute febrile illness with multi-organ dysfunction (mainly shock, acute renal failure and hemorrhage), bearing a mortality rate between 10 and 15% and affecting over 3,000 United Nation troops (Smadel J.E., 1953). Despite an enormous investigative effort by a special Hemorrhagic Fever Commission of the US Army, it was not until 1976 that Dr. H.W. Lee and colleagues discovered a virus-specific antigen in the lungs of a Korean striped field mouse or *Apodemus agrarius coreae*, which subsequently led to the isolation and characterization of the responsible agent in 1977 (Lee et al., 1978). This first prototype agent was called Hantaan virus, after the river Hantaan which runs near to the famous 38th parallel between North- and South Korea, where most of the battles were fought, but where also most of the cases were recorded and where the Hantaan virus-infected rodents were trapped.

The outbreak of HPS in the south western United States starting with a cluster of deaths in May 1993 and with a very high fatality rate in the initial outbreak, changed the recognized spectrum of hantavirus disease because the presentation was primarily a febrile illness complicated by the rapid development of respiratory failure. Renal and hemorrhagic manifestations were not pronounced. Within three weeks after the first casualties, some 24 suspect case-patients had been identified, most of them from New Mexico (MMWR, 2007b; MMWR, 2007a) and a few weeks later, the rodent *Peromyscus maniculatus* was identified as

the common and sole carrier of the agent (Childs et al., 1994). Genetic analysis done on rodent and human tissue, together with serological tests, demonstrated that a new virus most closely related to Prospect Hill virus and Puumala virus was the culprit (Feldmann et al., 1993; Nichol et al., 1993). This virus, finally named Sin Nombre, is the leading cause of HPS and several other hantaviruses vectored by other rodents in the New World occasionally induce similar disease. The emergence of HPS further increased world attention and research efforts on the diagnosis, control and treatment of hantavirus infections.

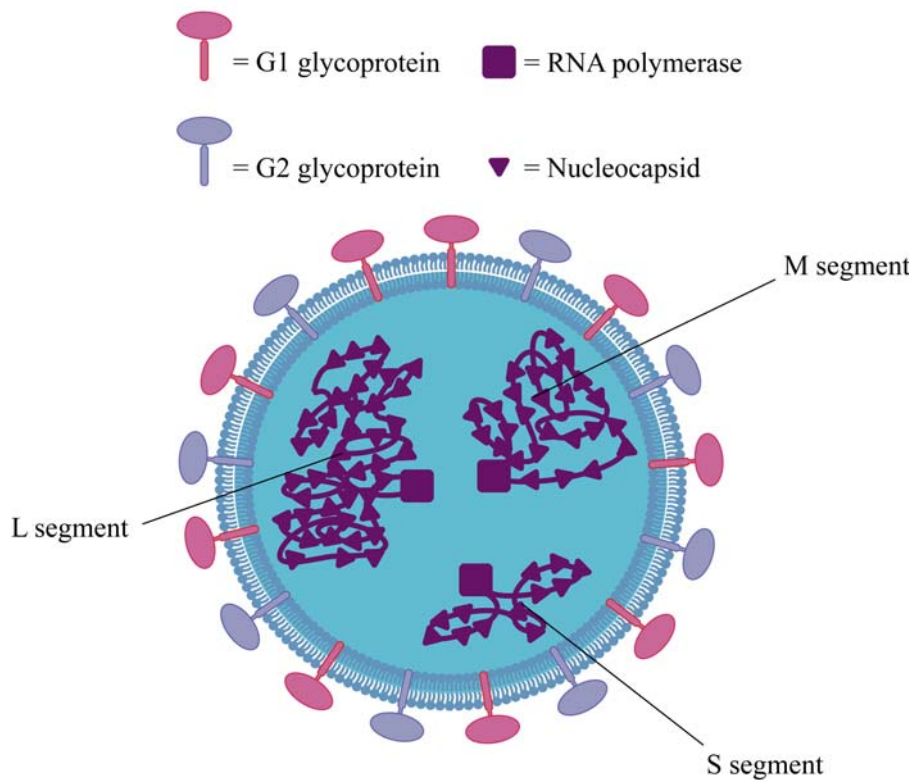


FIGURE 1.1: Graphical representation of a hantavirus particle.

### 1.3 Genome organization

Like all members of the family Bunyaviridae, the genome is negative sensed trisegmented and the hantavirus coding strategy is considered to be the simplest of the five genera. All three segments encode only one protein in the virus complementary sense. Although minor open reading frames have been noted in both virus sense and virus complementary sense, there is no evidence for protein products (Schmaljohn and Dalrymple, 1983; Schmaljohn et al., 1985). As with other Bunyaviridae, each of the three segments has a consensus 3'-terminal nucleotide sequence (AUCAUCAUC), which is complementary to the 5'-terminal sequence and is distinct from those of the other four genera. Such complementary sequences are

capable of forming pan handle structures, a consistent feature of the Bunyaviridae (Elliott et al., 1991). The pan handles probably serve an important role in viral transcription and replication similar to other viruses with this structure, such as vesicular stomatitis virus and influenza virus, whose transcription and replication strategies are better understood. The hantavirus genome consists of a large L segment (6530 to 6550 nt) coding for the viral RNA-dependent RNA polymerase, a medium M segment (3613 to 3707 nt) coding for a polyprotein cleaved by cotranslational processing to form the two viral envelope glycoproteins G1 and G2 and a small S segment (1696 to 2083 nt) coding for the nucleocapsid protein (Np) (Schmaljohn and Dalrymple, 1983) (FIGURE 1.1). There is no evidence of a nonstructural S segment (NSs) protein which is present in other genera of the Bunyaviridae. A short, 37 to 51 nt long, 5' non coding region (NCR) on the virus complementary strand is present in each of the three segments. A 3' NCR is also present: L segment 38 to 43 nt, M segment 168 to 229 and S segment 370 to 730 nt (Plyusnin et al., 1996). The 3' NCR of the S segment is conserved with regard to length and sequence within hantavirus species, suggesting some functional role. However, the length and sequence are very variable between species, except for the terminal pan handle sequence (Plyusnin et al., 1996).

#### **1.4 Hantavirus taxonomy and classification**

According to the current report of “The International Committee on Taxonomy of Viruses” (ICTV), the genus *Hantavirus* includes 22 virus species (TABLE 1.1) (Nichol et al., 2005). The ICTV guidelines to classify a hantavirus species consist of four rules. These rules are defined as follows: (i) a hantavirus species is found in a unique ecological niche, i.e. in a different primary rodent reservoir species or subspecies; (ii) a hantavirus species exhibits at least a 7% difference in amino acid identity when comparing the complete S segment and M segment sequences; (iii) a hantavirus species shows at least a fourfold difference in two-way cross-neutralization tests; (iv) hantavirus species do not naturally form reassortants with other species (Nichol et al., 2005). The 22 hantavirus species can be divided into different groups according to their primary rodent host subfamily and according to phylogenetic analysis. The first rodent-borne hantavirus to be isolated was Hantaan virus strain 76-118, isolated in 1976 out of the lungs of an *Apodemus agrarius coreae* (Lee et al., 1978) (FIGURE 1.2). Hantaviruses belonging to the same group as Hantaan virus are all carried by the Rodentia subfamily Murinae (Order Rodentia, Suborder Myomorpha, Superfamily Muriodea, Family Muridae). Shortly after the isolation of Hantaan virus, Puumala virus was isolated from a bank vole, *Myodes glareolus* (most often referred to as *Clethrionomys glareolus*) (Carleton et al., 2003;

Brummer-Korvenkontio et al., 1980). Puumala virus was the first member of a second group of hantaviruses, carried by the Rodentia subfamily Arvicolinae (Order Rodentia, Suborder Myomorpha, Superfamily Muriodea, Family Cricetidae).

**TABLE 1.1** List of hantavirus species as defined by ICTV.

<b>Hantavirus species</b>	<b>abbreviation</b>	<b>Rodent host</b>
<i>Andes virus</i>	ANDV	<i>Oligoryzomys longicaudatus</i>
<i>Bayou virus</i>	BAYV	<i>Oryzomys palustris</i>
<i>Black Creek Canal virus</i>	BCCV	<i>Sigmodon hispidus</i>
<i>Caño Delgadito virus</i>	CADV	<i>Sigmodon alstoni</i>
<i>Dobrava-Belgrade virus</i>	DOBV	<i>Apodemus flavicollis</i> <i>Apodemus agrarius</i>
<i>El Moro Canyon virus</i>	ELMCV	<i>Reithrodontomys megalotis</i>
<i>Hantaan virus</i>	HTNV	<i>Apodemus agrarius</i> <i>Apodemus peninsulae</i> <i>Niviventer confucianus</i>
<i>Isla Vista virus</i>	ISLAV	<i>Microtus californicus</i>
<i>Khabarovsk virus</i>	KHAV	<i>Microtus fortis</i>
<i>Laguna Negra virus</i>	LANV	<i>Calomys laucha</i>
<i>Muleshoe virus</i>	MULV	<i>Sigmodon hispidus</i>
<i>New York virus</i>	NYV	<i>Peromyscus leucopus</i>
<i>Prospect Hill virus</i>	PHV	<i>Microtus ochrogaster</i> <i>Microtus pennsylvanicus</i>
<i>Puumala virus</i>	PUUV	<i>Eothenomys regulus</i> <i>Myodes glareolus</i> <i>Myodes rufocanus</i>
<i>Rio Mamore virus</i>	RIOMV	<i>Oligoryzomys microtis</i>
<i>Rio Segundo virus</i>	RIOSV	<i>Reithrodontomys mexicanus</i>
<i>Seoul virus</i>	SEOV	<i>Rattus losea</i> <i>Rattus norvegicus</i> <i>Rattus rattus</i>
<i>Sin Nombre virus</i>	SNV	<i>Peromyscus leucopus</i> <i>Peromyscus maniculatus</i>
<i>Thailand virus</i>	THAIV	<i>Bandicota indica</i>
<i>Thottapalayam virus</i>	TPMV	<i>Suncus murinus</i>
<i>Topografov virus</i>	TOPV	<i>Lemmus sibiricus</i>
<i>Tula virus</i>	TULV	<i>Microtus arvalis</i> <i>Microtus rossiaemeridionalis</i>

Hantaviruses carried by both subfamilies, Arvicolinae and Murinae, can be the causative agents of HFRS in the Old World. After the major outbreak of HPS in the United States in May 1993, Sin Nombre virus, a first member of a third group of hantaviruses, was isolated out of the lungs of a *Peromyscus maniculatus* or North American Deermouse (Nichol et al., 1993). Members of this third group are carried by the Rodentia subfamilies Neotominae and Sigmodontinae (Order Rodentia, Suborder Myomorpha, Superfamily Muroidea, Family Cricetidae) and are responsible for HPS in the New World. In addition two New World hantaviruses, Prospect Hill virus (Lee et al., 1982) and Isla Vista virus (Song et al., 1995), are carried by *Microtus pennsylvanicus* and *Microtus californicus* respectively (Rodentia subfamily Arvicolinae) but have not been associated with human disease so far.



**FIGURE 1.2:** From left to right: *Myodes glareolus* or bank vole, *Apodemus flavicollis* or yellow-necked field mouse, and *Apodemus agrarius* or striped field mouse.

It is noticeable that, Seoul virus, an Old World hantavirus carried by *Rattus rattus* and *Rattus norvegicus*, has been isolated from wild rats in the New World as well, even before the 1993 HPS outbreak (LeDuc et al., 1984; LeDuc et al., 1985; Tsai et al., 1985). This might explain why the very first reports of symptomatic hantavirus infections in the New World were in fact descriptions of Seoul virus-induced HFRS cases, first in Recife, Brazil, and then in the USA (Hindrichsen et al., 1993; Glass et al., 1994). Interestingly, the rodent host of Sin Nombre virus, *Peromyscus maniculatus*, is habitually referred to as a species of the subfamily Sigmodontinae, but in fact *Peromyscus* represents a genus of the subfamily Neotominae (Musser and Carleton, 2005). Thottapalayam virus, isolated from a *Suncus Murinus* or Asian house shrew back in 1964, was first believed to be an arbovirus (Carey et al., 1971). When it was found to have bunyavirus morphology and the unique and conserved 3' terminal nucleotide signature carried by all hantaviruses, Thottapalayam virus joined the hantavirus genus where it is the sole member of a fourth, very distinct group. Very recently, Tanganya

virus was found in a *Crocidura theresae* or Therese's Shrew in Guinea. It is probably the first member of a fifth very distinct group of hantaviruses (Klempa et al., 2007).

### **1.5 Epidemiology of hantavirus infections**

Infectious febrile diseases with hemorrhagic and renal manifestations have been recognized across the Eurasian continent for more than 50 years. Seven thousand cases of a "war nephritis", clinically similar to nephropathia epidemica (NE), were reported among British soldiers stationed in Flanders during World War I. Nevertheless, these disorders were not given much attention by Western physicians until the early 1950's during the Korean conflict, when over 3000 cases were diagnosed among United Nations forces. Subsequently, it was recognized that Korean hemorrhagic fever and clinically similar diseases, collectively termed hemorrhagic fever with renal syndrome (HFRS), pose a significant health threat in much of Asia and parts of Europe and Scandinavia. Non-pathogenic infection of rodent populations apparently provides a reservoir for the causative agent. Infection of humans occurs via aerosol from rodent urine, feces, and saliva. Mortality rates have decreased from the 10 to 15% seen during the Korean conflict to 5% or less, if improved fluid and electrolyte management and/or renal dialysis are available. While around 2,500 hantavirus cardiopulmonary syndrome (HPS) cases have been reported so far, approximately 150,000 HFRS cases are estimated to occur worldwide annually. An estimated 50,000 to 100,000 cases occur annually in the People's Republic of China alone, with mortality rates ranging from 5 to 20%, in various provinces (Chen et al., 1986). In Europe, Dobrava-Belgrade virus (DOBV) seems to be the most virulent hantavirus with a fatality rate up to 12%, mainly due to severe hemorrhagic manifestations (Avsic-Zupanc et al., 1992). Puumala virus (PUUV), which causes the milder disease NE, has a fatality rate that varies from 0.1 to 0.3 % (Brummer-Korvenkontio et al., 1999). For Belgium, more than 1,740 confirmed NE cases are reported in the period 1985-2006 (W.I.V. G. Ducoffre).

## **2. HUMAN HANTAVIRUS DISEASE AND PATHOGENESIS**

Both HFRS and HPS are associated with acute thrombocytopenia and changes in vascular permeability and both diseases may have pulmonary or renal symptoms (Klempa et al., 2004; Linderholm and Elgh, 2001; Nolte et al., 1995). Hantaviruses cause diseases in humans and both pathogenic and non-pathogenic hantaviruses have the same tissue tropism, replicating predominantly in endothelial cells and macrophages (Yanagihara and Silverman, 1990).

Although less distinct in HPS than in HFRS, the clinical course can be divided into five distinct phases and viremia is thought to occur subsequent to infection of alveolar macrophages, leading to infection of kidney and lung endothelial cells where hantaviruses replicate (Lednicky, 2003; Yanagihara and Silverman, 1990). A first phase in human hantavirus disease, the febrile phase, occurs 2-4 weeks after infection, with an abrupt onset of disease with high fever, chills, malaise, headache, and after the second day of onset, gastrointestinal symptoms, vomiting and abdominal pain, normally lasting about 3 to 7 days. Lumbal pain caused by renal swelling, often announces renal involvement in HFRS. In some HFRS cases, conjunctival hemorrhages and fine petechiae occur at the end of this phase, together with (often massive) proteinuria. A characteristic drop of the blood platelet number (thrombocytopenia) is the beginning of the second phase, the hypotensive phase both in HFRS and HPS, lasting from several hours to 2 days. At this stage, some cases of HFRS and HPS die of irreversible shock. In the third phase, the oliguric phase of HFRS, lasting about 3 to 7 days, anuria and renal failure define severity in HFRS, whereas in HPS acute lung edema can rapidly worsen in this stage, prompting mechanical ventilation. The beginning of the fourth, diuretic phase is a positive sign for the patient. Diuresis of 3 to 6 liter/24 hours is observed. The last phase, the convalescent phase is characterized by slow normalization of the clinical markers and recovery of the patient during several weeks (Lahdevirta, 1971). It is noteworthy also that the non-specific prodrome leading to acute cardiopulmonary deterioration in HPS can be confused with those of mycoplasmal and chlamydophilial infections, as well as those of leptospirosis, Legionnaire disease, Q fever, septicemic plague, tularemia, coccidiomycosis, and histoplasmosis for HFRS.

The cellular entry of pathogenic hantaviruses is mediated by  $\beta_3$ -integrins ( $\alpha_v\beta_3$ -integrin receptor) (Gavrilovskaya et al., 1998). These integrins are heterodimeric receptors composed of a and b subunits that mediate cell-to-cell adhesion and platelet aggregation (Phillips et al., 1988). Endothelial cells and platelets are prominent regulators of vascular functions and integrins play key roles in barrier functions of these cells (Gavrilovskaya et al., 1998; Peters and Khan, 2002). Hantaviruses that are thought to be non-pathogenic seem to use another yet unidentified integrin receptor (Mackow and Gavrilovskaya, 2001). The occurrence of large intracellular inclusion bodies, likely to contain viral nucleocapsid protein, is typical for hantavirus infections (Lee and Cho, 1981). Although hantaviruses have been shown to replicate in cultured human endothelial cells, there is considerable evidence that immune mechanisms rather than direct viral cytopathology, are responsible for the principal abnormality, vascular dysfunction resulting in plasma leakage in HFRS and HPS (Cosgriff,

1991; Zaki et al., 1995). The lack of suitable animal models for most of the frequently occurring hantaviruses is a significant obstacle in the understanding of hantavirus disease pathogenesis.

### **3. LABORATORY DIAGNOSIS OF HANTAVIRUS INFECTIONS**

Several studies have demonstrated the production of Np-specific antibodies during the acute phase of the disease, whereby detection of Np-specific IgM antibodies in clinical samples appeared to be a good indicator of a recent hantavirus infection (Clement et al., 1995; Vapalahti et al., 1995). The detection of hantavirus-specific IgM using recombinant Nps in an ELISA format, is by far the most valuable and widely used test for diagnosing acute phase hantavirus infections (Padula et al., 1998; Li et al., 2002). Truncated recombinant Nps were shown to be even more specific, moreover in most cases specific enough to differentiate the involved hantavirus serotype (Araki et al., 2001; Elgh et al., 1997). The plaque reduction neutralization test is considered to be the gold standard serological test as reliable diagnostic test for hantavirus serotype identification involved in the infection. Reverse transcriptase PCR with type-specific primers has been shown to be a useful tool in the diagnosis of hantavirus (Horling et al., 1995; Nichol et al., 1993; Terajima et al., 1999; Xiao et al., 1991), although hantaviral RNA is only detected in the first days after onset of disease in NE (Plyusnin et al., 1997). Sin Nombre virus RNA could be detected up to ten days after onset of disease in serum specimens of HPS patients (Terajima et al., 1999). When using quantitative reverse transcriptase PCR, it is possible to detect less than 10 TCID<sub>50</sub> mL<sup>-1</sup> Puumala virus RNA (Garin et al., 2001). Immunochromatographic assays for rapid and reliable laboratory diagnosis of hantavirus infection are more commonly used in recent years (Hujakka et al., 2003; Sirola et al., 2004). These tests have the same sensitivity as ELISA assays; in some cases they had even a higher sensitivity. The immunochromatographic rapid tests gave relatively low serological cross-reactivity between Dobrava-Belgrade virus, Hantaan virus and Puumala virus (Hujakka et al., 2003), which makes these tests a good alternative for the time-consuming ELISA assays.

#### 4. PREVENTION OF HANTAVIRUS INFECTIONS

##### 4.1 *Vaccine strategies against hantaviruses*

A variety of vaccines has been developed using both inactivated virus and recombinant DNA technology. Several groups throughout Asia have made inactivated hantavirus vaccines. Most of these vaccines were made using formalin inactivated rodent brain-derived virus (Cho and Howard, 1999; Yamanishi et al., 1988), similar to those used to prepare Japanese encephalitis virus and rabies vaccines (Acha, 1967; Gupta et al., 1991). A commercial Korean inactivated Hantaan virus vaccine, named Hantavax<sup>TM</sup>, was shown to be effective in protecting mice and humans from HFRS (Cho and Howard, 1999; Cho et al., 2002; Hjelle, 2002). After three vaccinations, ELISA antibody seroprevalence was 100% in the study group. In the neutralization assay however, only 50% of vaccine recipients possessed measurable neutralizing antibodies after three vaccinations. A significant proportion of volunteers showed high hantavirus-specific IgG ELISA titers in the absence of detectable neutralizing antibodies (Cho and Howard, 1999; Cho et al., 2002). Another study found a neutralization response in only 33% of recipients after two immunizations (Sohn et al., 2001). The absence of any serious adverse event despite the use of millions of doses during the last 10 years suggests that this formalin-inactivated vaccine is safe. Cell culture-based hantavirus vaccines are the basis of several vaccines like influenza virus vaccine and polio virus vaccine (Merten et al., 1996; Montagnon et al., 1981). Both Hantaan virus and Seoul virus cell culture-based vaccines, prepared in golden hamster kidney cells, Mongolian gerbil kidney cells or Vero E6 cells, were shown to induce fewer side effects and provided a more effective immunity, with high levels of neutralizing antibodies, in comparison to rodent brain-derived vaccines (Choi et al., 2003; Hjelle, 2002; Hooper and Li, 2001; Lu et al., 1996; Song et al., 1992). Both the rodent brain-derived vaccines and the cell culture based vaccines, yielded only low levels of ELISA antibody titers as well as neutralizing antibody titers one year after vaccination, raising questions about the long-term efficacy of these vaccines. Several techniques have been used for the development of vaccines by expressing hantavirus proteins using recombinant DNA technology. Recombinant hantaviral antigens have been expressed in insect cells, mammalian cells, *E. coli* and transgenic plants. Recombinant hantavirus G1 and G2 glycoproteins, and Np expressed in several systems, have proven protective potential (Dargeviciute et al., 2002; de Carvalho et al., 2002; Geldmacher et al., 2004; Khattak et al., 2002)). To overcome problems of low immunogenicity of monomeric viral proteins, virus-like particles (VLPs) can be used as immunogens. VLPs are non-infectious, highly-structured, repetitive protein complexes that resemble the structural and immunological properties of the

original virus particles. Ulrich and colleagues have successfully achieved protection in a bank vole model using Hepatitis B virus core-derived chimeric particles carrying a 45 amino acid segment of the Puumala virus Np (Koletzki et al., 2000; Ulrich et al., 1998). This vaccine approach is promising due to the high immunological response these VLPs provoke (Pumpens and Grens, 2001). Inoculation with live recombinant virus, like vaccinia virus and CMV, showed to be effective in protecting animals from challenge with hantavirus (Chu et al., 1995; McClain et al., 2000; Rizvanov et al., 2003; Schmaljohn et al., 1992; Terajima et al., 2002; Xu et al., 1992). A major disadvantage of this method is pre-existing immunity against the carrier virus in people who need to be vaccinated. In a phase I clinical safety trial with a recombinant vaccinia vaccine expressing both the Hantaan virus Nps and G1/G2 glycoproteins, only 50% of the volunteers with a pre-existing immunity against vaccinia, exhibited neutralizing antibody responses after two vaccinations, whereas 100% of the vaccinia-naïve volunteers exhibited neutralizing antibody responses after the second vaccination (McClain et al., 2000). Naked DNA vaccines, usually based on plasmid DNA, have been demonstrated to be promising vaccine approaches for various viral infections (Duenas-Carrera, 2004; Giri et al., 2004; Kim et al., 2004; Lodmell et al., 2003). Several groups have utilized the whole Np and the G1/G2 glycoproteins of Andes virus, Hantaan virus, Puumala virus, Seoul virus and Sin Nombre virus as immunogens, mostly driven by a mammalian RNA polymerase 2 promotor (Bharadwaj et al., 1999; Custer et al., 2003; Hjelle, 2002; Hooper and Li, 2001; Hooper et al., 2001; Kamrud et al., 1999; Koletzki et al., 2001). These plasmids have been shown to offer protection against experimental infection in hamster, mouse or macaque models. Constructs expressing the G1 and G2 glycoproteins exhibited the desired neutralizing antibody responses. Sindbis virus replicons have also been evaluated as a possible DNA-based vaccine approach. But both Sindbis virus and packaged Sindbis virus replicons encoding for either the M or S segment of Seoul virus did not exhibit a protective immune response in Syrian hamsters (Kamrud et al., 1999).

#### **4.2 Antiviral therapy**

Currently, no WHO-recognized antiviral drug or immunotherapeutic agent is available for treatment of the hantavirus diseases. Ribavirin (1- $\beta$ -D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide) has been shown to have *in vitro* activity and to some extent also *in vivo* activity against some hantaviruses (Severson et al., 2003). Huggins and colleagues (Huggins et al., 1986) studied the efficacy of ribavirin therapy given to Hantaan virus-infected suckling mice on days 6 through 20 after infection. The ribavirin-treated mice had a higher survival rate than

the placebo control group. Based on these findings, Huggins' group conducted a double-blind placebo-controlled trial with HFRS patients in several countries including China and Korea, showing a sevenfold reduction in morbidity in the ribavirin-treated group (Huggins et al., 1991). A statistically significant reduction in fatal outcomes from 10 out of 117 patients receiving placebo to 3 out of 125 patients receiving ribavirin was reported. The results of a nonselective open-label trial in patients suffering from HPS however, showed disappointing results (Chapman et al., 1999).

Vero E6 cells, pre-treated with human interferons IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$  resulted in dose-dependent inhibition of Hantaan virus replication. Of the three human interferons, IFN- $\beta$  inhibited virus replication most effectively (Tamura et al., 1987). Newborn mice treated with IFN- $\beta$  before infection with Hantaan virus, showed a survival rate of 85 to 90% against less than 20% for non treated mice. The same observations were described for Puumala virus and Tula virus. Human MxA protein, a type I interferon-inducible intracytoplasmic protein, mediates antiviral actions against several members of the family *Bunyaviridae* after interferon stimulation (Frese et al., 1996). The human MxA protein has the capacity to inhibit Puumala virus and Tula virus replication, and RNA accumulation in virus-infected Vero E6 cells (Kanerva et al., 1996). Kraus and colleagues studied the kinetics of MxA protein expression in Hantaan virus and Tula virus infections. Tula virus-infected HUVECs showed an early onset (16h post infection) of MxA protein expression, whereas Hantaan virus-induced MxA proteins appeared relatively late (48h post infection). Viral titers produced by Tula virus-infected cells were found to be much lower than those produced by Hantaan virus-infected cells. On Vero E6 cells, which lack IFN- $\alpha/\beta$  genes, Tula virus could grow as efficiently as Hantaan virus (Kraus et al., 2004). Interestingly, pathogenic and non-pathogenic hantaviruses regulate endothelial cell responses differently. Hantaan virus and New York virus seem to be able to postpone interferon stimulated genes synthesis in comparison with the non-pathogenic Prospect Hill virus. This short period of delay seems to be enough to escape antiviral processes induced by INF. Replication of Hantaan virus and New York virus is still effective even in the presence of high levels of the MxA protein (Geimonen et al., 2002). These findings suggest that pathogenic hantaviruses, in contrast to non-pathogenic hantaviruses, are able to block the early antiviral immune response in human endothelial cells (Geimonen et al., 2002; Kraus et al., 2004).

Tragacanthin polysaccharides from *Astragalus brachycentrus* and *Astragalus echidnaeformis* plants have been suggested as a potential therapeutic approach to hantavirus disease as these

compounds have been shown to have antiviral activity against Punta Toro virus (a phlebovirus member of the family *Bunyaviridae*, used as a model for studying the treatment of hantavirus infections) *in vitro* and *in vivo* (Smee et al., 1996).

#### **4.3 Rodent control**

Potentially the most effective way of controlling hantavirus disease is by limiting contact with rodents and their excrement. Simple rodent proofing measures to dwellings have been shown to eliminate or substantially reduce exposure to *Peromyscus maniculatus* (Glass et al., 1997). Similarly working practices and conditions in agriculture, forestry and the military should be modified where possible to reduce human rodent exposure. General precautions for residents living in affected areas have been produced (MMWR, 1993) and deal with the elimination of rodents inside the home, prevention of rodents from entering the home and reducing rodent food and shelter near homes. Guidance for hikers and campers has also been created (Warner, 1996).

In laboratory animal facilities, all laboratory work involving the propagation of hantaviruses in cell culture or animals should be conducted in biosafety level 3 conditions. Generally high standards of animal husbandry and adherence to safety protocols must be used when dealing with experimental animals. Even in work with animals not known to be infected with hantavirus, protocols should minimize potential contact with secretions.

#### **5. AIMS AND OBJECTIVES**

Thirty years after its discovery, there is still no safe and effective vaccine or antiviral treatment against hantavirus infections. The main objective of this research project is to develop several recombinant proteins derived from the nucleocapsid proteins of Dobrava virus and Puumala virus, and to determine their immunological and protective properties *in vivo* by using a mouse outbred NMRI model. Because of the lack of a lethal mouse model for both viruses, a new neutralization test, the “replication reduction neutralization test” (RRNT) was developed to score protection against hantavirus challenge in immunized mice. An enzyme-linked immunosorbent assay (ELISA) based on a truncated recombinant protein derived from the nucleocapsid proteins of Dobrava virus and Puumala virus was developed to characterize the immunological properties of the recombinant Dobrava virus and Puumala virus nucleocapsid proteins. In line with the search for an effective vaccine or antiviral

treatment against hantavirus infections, chloroquine was tested *in vitro* and *in vivo* for its antiviral potential against hantaviruses.

A second research area involves the genetic classification of hantaviruses. To date, hantaviruses are demarcated into species based on the guidelines of the International Committee on Taxonomy of Viruses (ICTV). However, several publications have recently proposed new hantavirus species based on only a single criteria, such as a distinct rodent host, or the absence of related human disease, without considering strictly the ICTV guidelines for species demarcation. Complete sequences derived from the S, M and L segment (as found in Genbank) were used to obtain an objective and straightforward genetic classification as a modern alternative to the current ICTV classification of hantaviruses.



**HANTAVIRUS GENETIC CLASSIFICATION BASED ON  
NUCLEOCAPSID PROTEIN SEQUENCES**

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This chapter is submitted to:

**Piet Maes, Boris Klempa, Jan Clement, Jelle Matthijnsens, Daniel C. Gajdusek, Detlev H. Krüger, and Marc Van Ranst.** 2007. Hantavirus genetic classification based on nucleocapsid protein sequences. *Molecular Phylogenetics and Evolution* submitted.

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## 1. SUMMARY

Hantaviruses are demarcated into species based on the guidelines provided by the International Committee on Taxonomy of Viruses (ICTV), which are rather difficult to comply and therefore often ignored by the descriptors of novel hantavirus strains. To obtain an objective and straightforward genetic classification as a modern alternative to the current ICTV classification, we analyzed 218 complete S segment sequences, 92 complete M segment sequences and 29 complete L segment sequences by using similarity comparison, together with maximum likelihood and neighbor-joining analysis. Based on these analyses, we defined guidelines concerning the genetic classification of hantaviruses. Using S segment amino acid sequences (but not nucleotide sequences) allowed efficient demarcation between different hantavirus species and lead to new criteria for demarcation of groups (amino acid distance >24%) and species (amino acid distance >10%). For the M segment, these criteria were set on a distance greater than 32% for group demarcation and a distance greater than 12% for species demarcation respectively. For the L segment, only 29 complete sequences were available in Genbank, which was insufficient to draw well-corroborated conclusions. Within this study, five different groups and 18 virus species could be delineated, and by using maximum likelihood and neighbor-joining, several strains could be identified. In conclusion, we propose a novel genetic classification scheme of the genus Hantavirus. This tentative and novel genetic classification method might provide an easy alternative to the demanding ICTV criteria for the classification of hantaviruses, especially for those viruses discovered without any isolate.

## 2. INTRODUCTION

According to the current report of ICTV, the genus *Hantavirus* includes 22 virus species. A significant part of them, particularly the New World hantaviruses, have been characterized only by molecular techniques. The culturing of hantavirus isolates is often a problem. Moreover, for many laboratories, performing neutralization tests (as a criterion requested by ICTV) is a major problem, since these tests usually require biosafety level 3 facilities (BSL-3). With the current international safety rules concerning transportation of biohazard material, it can be hard to acquire the reference strains needed for these compulsory neutralization tests. Therefore, researchers often classify their hantavirus strain based on phylogenetic tests

performed on sequences of one or more hantavirus segments. Unfortunately, no generally accepted rules are provided so far to classify hantaviruses genotypically.

The current ICTV guidelines consist of four rules to characterize a hantavirus species. The rules are defined as follows: (i) a hantavirus species is found in a unique ecological niche, i.e. in a different primary rodent reservoir species or subspecies; (ii) a hantavirus species exhibits at least a 7% difference in amino acid identity when comparing the complete S segment and M segment sequences; (iii) a hantavirus species shows at least a fourfold difference in two-way cross-neutralization tests; and (iv) hantavirus species do not naturally form reassortants with other species (Nichol et al., 2005). Several reasons can be put forth to question these ICTV rules, and adapt them better to the current situation in the hantavirus field:

(i) It is characteristic for hantaviruses that they are closely linked to their rodent hosts. Specificity of the rodent host is the main factor in explaining the geographical spread of human hantavirus disease and the mechanisms of an outbreak or even a single sporadic occurrence. However, the rather easy paradigm of “one hantavirus species for one rodent species” is no longer supported, since it has been shown that several distinct hantaviruses are carried by the same rodent host: e.g. *Apodemus agrarius* can be the host for at least two clearly different hantavirus species being Hantaan virus and Dobrava-Belgrade strain B viruses; the South American hantaviruses, Black Creek Canal virus and Muleshoe virus, are both carried by the rodent host *Sigmodon hispidus* (Powers et al., 1999). Moreover, several hantavirus species like Andes virus, Hantaan virus, Dobrava-Belgrade virus, Puumala virus and some others have been isolated out of at least two different rodent species (TABLE 1).

(ii) Several ICTV defined hantavirus species are not in agreement with the second rule of the ICTV classification which defines a difference in identity of at least 7% for both the S segment and M segment amino acid sequences. This is the case for Khabarovsk virus and Topografov virus, and several South American species. Moreover, there are several different ways to calculate percentage identity (PID) and each may yield a different result for the same alignment (Raghava and Barton, 2006). According to Raghava and colleagues, the maximum variation in PID due to the calculation method was 11.5% when using the identical alignment (Raghava and Barton, 2006). Using PID for sequence similarity is a very crude method for scoring. It is much better to use a method that takes account of the length and composition of the sequences as well as including scores for non-identical amino acids.

(iii) Although a single amino acid substitution in the major neutralizing epitope could theoretically allow a virus to escape neutralization, up to date, a neutralization test is, for obvious reasons, the main criterion to define a hantavirus species. However, hantaviruses are

often initially discovered by molecular techniques, thus without a virus isolate. Moreover, hantaviruses are notoriously difficult to grow in cell culture. Even with a virus isolate, it must be scored against a complete panel of hantavirus reference strains, which only a small number of laboratories is capable of performing. Moreover, *in vitro* experiments with most hantaviruses, must be performed under biosafety level 3 environment because of the hazardous nature of these viruses.

(iv) The last rule of ICTV asserts that hantavirus species may not naturally form reassortants with other species. However, several studies have reported the occurrence of reassortments between distinct hantavirus species at least in cell culture (Rodriguez et al., 1998; McElroy et al., 2004; Rizvanov et al., 2004). More important, demonstration of the non-existence of reassortants in nature can be never absolutely provided. In other words, this rule would require an “evidence of non-existence”. We are inclined to believe, therefore, that the last rule of the ICTV classification guidelines (absence of natural occurrence of reassortants) is not useful.

With this study we attempt to provide general rules for a genetic classification based on the nucleocapsid protein of hantaviruses, with respect to the current ICTV classification for hantavirus species.

### **3. MATERIALS AND METHODS**

#### **3.1 *Hantavirus sequences***

Out of 1783 hantavirus sequences found in Genbank, submitted prior to June 2007, 218 complete S segment sequences (TABLE 2.2), 92 complete M segment sequences and 29 complete L segment sequences were selected. The dataset of 218 S segment sequences was checked for identical amino acid sequences using DAMBE v4.2.13 software (Xia and Xie, 2001), and finally, 185 unique and complete S segment sequences were identified and selected. Several hantaviruses were not included in the initial analysis, because only partial S segment sequences or no S segment sequences at all were available in Genbank. The latter hantaviruses include Anajatuba virus, Araraquara virus, Blue River virus, Calabazo virus, Castelo dos Sonhos virus, Central Plata virus, Jabora virus, Juquitiba virus, Limestone Canyon virus, Newfound Gap virus, Rio Mearim virus, Taimyr virus, Tanganya virus and several strains of Dobrava-Belgrade virus, Hantaan virus, Puumala virus, and Seoul virus.

### **3.2 Similarity calculation**

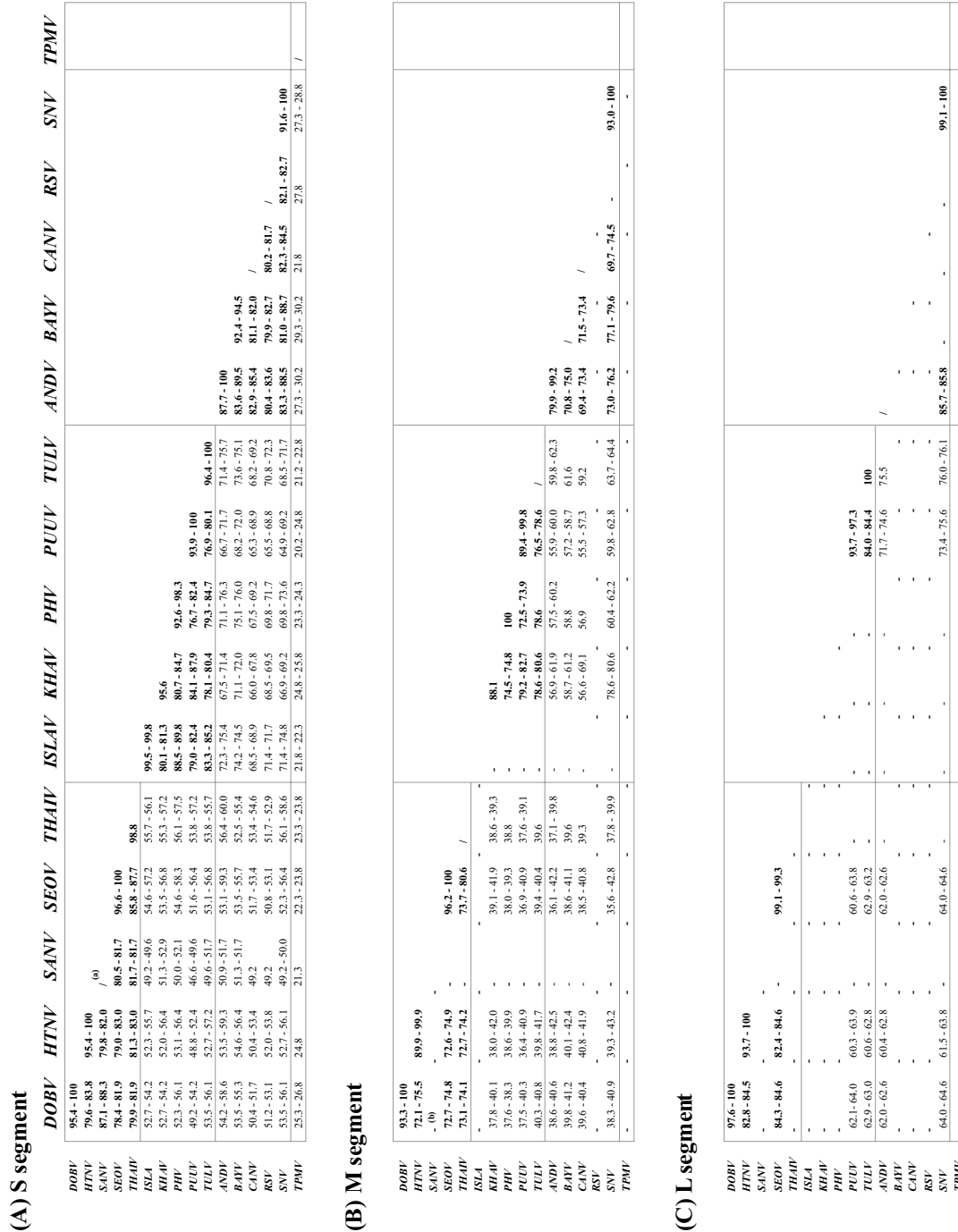
Nucleotide and amino acid sequences of the three hantavirus segments were aligned using MUSCLE v3.6 (Edgar, 2004). The alignments were formatted and corrected manually by using the multiple sequence alignment editor GeneDoc v2.6.002 and similarities were calculated with Mega v3.1 software (Kumar et al., 2004) by using the Kimura 2-parameter and the Poisson correction model for nucleotide and amino acid sequences respectively. The similarities were sorted into three categories: *variant*, similarities between different variants of the same hantavirus species; *species*, similarities between different hantavirus species within the same hantavirus group; and *genogroup*, similarities between different hantavirus species across hantavirus groups. These data were used to generate histograms of the distributions of similarities.

### **3.3 Neighbor-joining and maximum likelihood analysis**

Neighbor-joining trees were generated using Mega v3.1 and bootstrap analysis based on 10000 replicates was used to estimate the statistical support for the branching pattern. Maximum likelihood trees were calculated by the heuristic search method with branch swapping by TBR with PAUP v4.0b10 software to check the consistency of the tree topology between the maximum likelihood and neighbor-joining methods. For the maximum likelihood analysis, MODELTEST v3.06 (Posada and Crandall, 1998) was used to identify the evolutionary model that best fits the data; the phylogenetic model GTR+G+I was selected to calculate the likelihood parameters, with a gamma distribution shape of 0.617354.

### **3.4 Bootscanning analysis**

Bootscanning analysis was performed using window sizes of different lengths with a step size of 10 amino acids (Salminen et al., 1995). The frames were generated from the complete S and M segment amino acid alignments using ClustalW (Thompson et al., 1994) and bootstrap analysis based on 1000 replicates was performed by using the PAUP v4.0b10 software. Similarity plots were constructed with window sizes of different lengths with a step size of 10 amino acids by using the Poisson correction model.

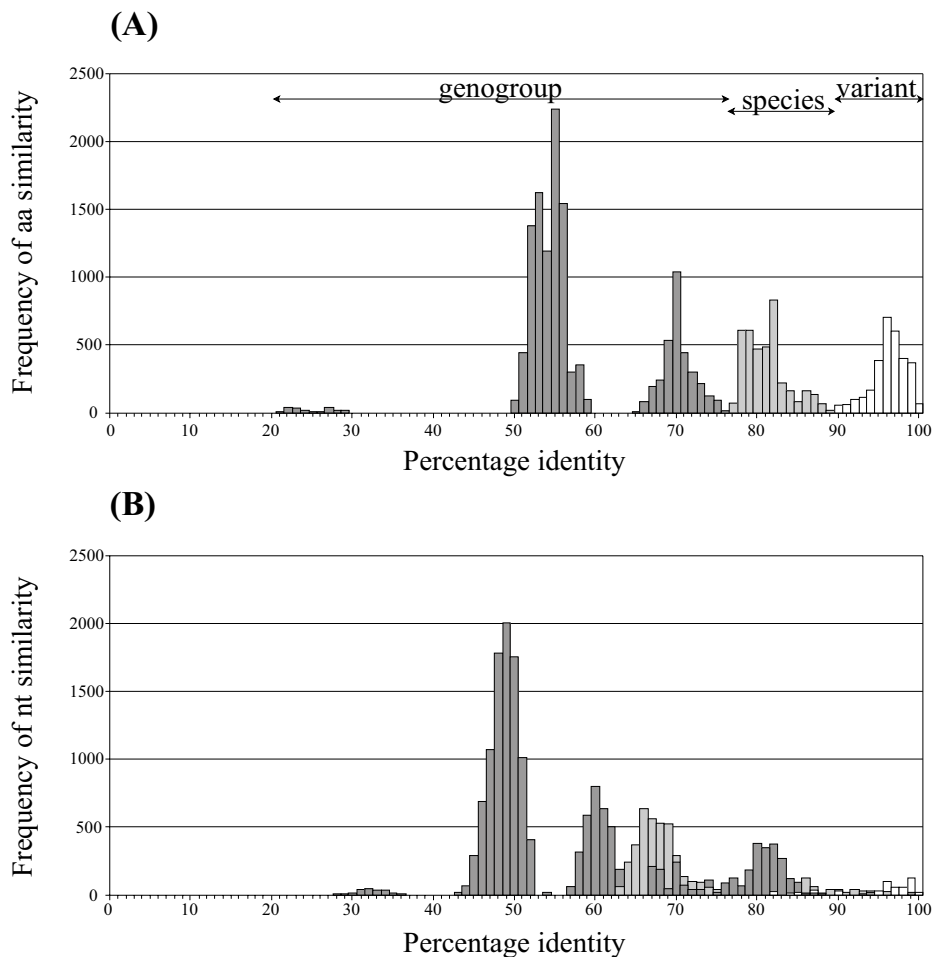


**FIGURE 2.1:** Percent amino acid sequence similarity ranges for complete sequences of (A) S segment, (B) M segment, and (C) L segment. Group 1: DOBV = Dobrava-Belgrade virus, HTNV = Hantaan virus, SANV = Sangassou virus, SEOV = Seoul virus, THAIV = Thailand virus; Group 2: ISLA = Isla Vista virus, KB-TP = Khabarovsk-Topografov virus, PHV = Prospect Hill virus, PUUV = Puumala virus, TULV = Tula virus; Group 3: ANDV = Andes virus, BAYV = Bayou virus, BCCV = Black Creek Canal virus, CANV = Caño Delgado virus, EMCV = El Moro Canyon virus, LNV = Laguna Negra virus, MULV = Muleshoe virus, RMV = Rio Mamore virus, RSV = Rio Segundo virus, SNV = Sin Nombre virus; and Group 4: TPMV = Thottapalayam virus.

## 4. RESULTS

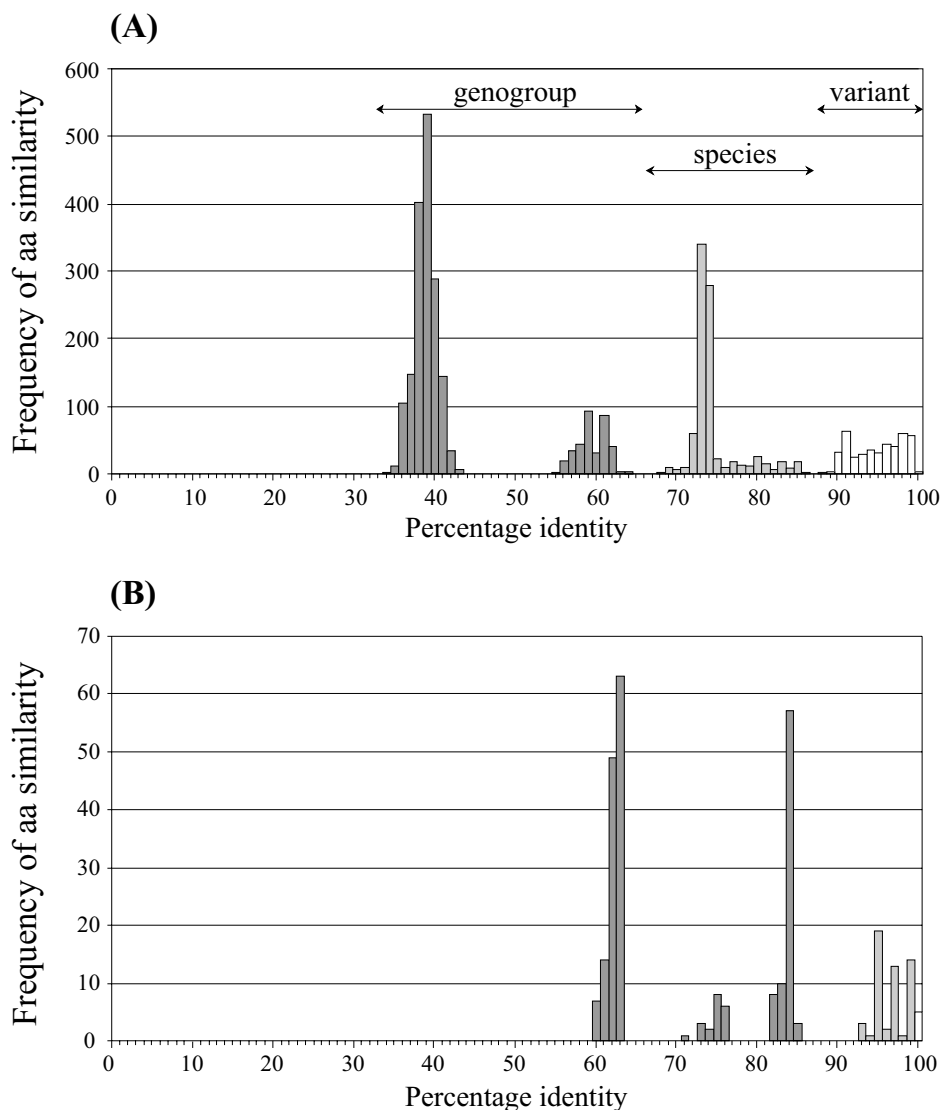
### 4.1 Similarity distribution of hantaviruses

Similarity percentages between sequences were calculated (FIGURE 2.1) and the frequency distribution of these similarities was plotted (percentage of similarity versus the frequency) into histograms (FIGURE 2.2 and FIGURE 2.3). The amino acid similarities calculated using Poisson correction from the three segments showed four major non-overlapping peaks. The histograms based on the nucleotide similarity ranges of the three segments also yielded some major peaks, however, the similarity ranges overlapped (FIGURE 2.2B). For this reason, no conclusion could be made based on the nucleotide similarities.



**FIGURE 2.2:** (A) Frequency distribution of similarities derived from the complete S segment amino acid alignment. The similarity ranges can be divided in *variant*, *species* and *genogroup* (from light to dark gray respectively). The genogroup similarities can be divided in similarity ranges between groups 1, 2 and 3 versus Thottapalayam virus, group 1 versus group 2 and 3, and group 2 versus group 3. (B) Frequency distribution of similarity from the complete S segment nucleotide alignment. The histogram shows some major peaks, but the similarity ranges overlap. G1: group 1, G2: group 2, and G3: group 3. aa: amino acids; nt: nucleotides.

For the S segment amino acid sequences, using the distance matrixes (FIGURE 2.1), the histogram analysis (FIGURE 2.2A) and a phylogenetic tree based on neighbor-joining analysis (FIGURE 4), we calculated and sorted the amino acid similarity ranges into three categories: (a) *genogroup*: the similarity ranges between hantavirus groups was 50.0 to 75.9%, without Thottapalayam virus. The similarity range of Thottapalayam virus against the three other groups was 20.7 to 30.2%. (b) *species*: the similarity range between different hantavirus species within the same hantavirus group was 76.0 to 89.9%. (c) *variant*: the similarity range between different isolates of the same hantavirus species was between 90.0 and 100%.



**FIGURE 2.3:** (A) Frequency distribution of similarities from the complete M segment amino acid alignment. The similarity ranges can be divided in *variant*, *species* and *genogroup* (from light to dark gray respectively). (B) Frequency distribution of similarities from the complete L segment amino acid alignment. The number of complete L segment sequences, available in Genbank to date (25 complete L segment sequences) is insufficient to draw any conclusions. aa: amino acids.

For the M segment, the similarity ranges were set on 34.0 to 67.9%, 68.0 to 87.9%, and 88.0 to 100%, for *genogroup*, *species* and *variant* respectively. For the L segment, only 29 complete sequences were available in Genbank, which was insufficient to draw well-corroborated conclusions.

This differentiation in *genogroup*, *species* and *variant* was possible for at least the S and M segments. The high number of 185 complete and unique S segment sequences available in Genbank made the S segment the pre-eminent hantavirus segment to use as a basis for a genetic classification (FIGURE 2.2).

When calculating nucleotide similarities based on alignments consisting of the coding region of the S segment, considering the first and second base of each codon only, the histograms based on these similarities showed clear peaks with almost no overlap. The similarity ranges could be set on 35.4 to 68.0-70.2%, 68.0-70.2 to 77.0-80.9%, and 77.9-81.0% to 100%, for *genogroup*, *species* and *variant* respectively.

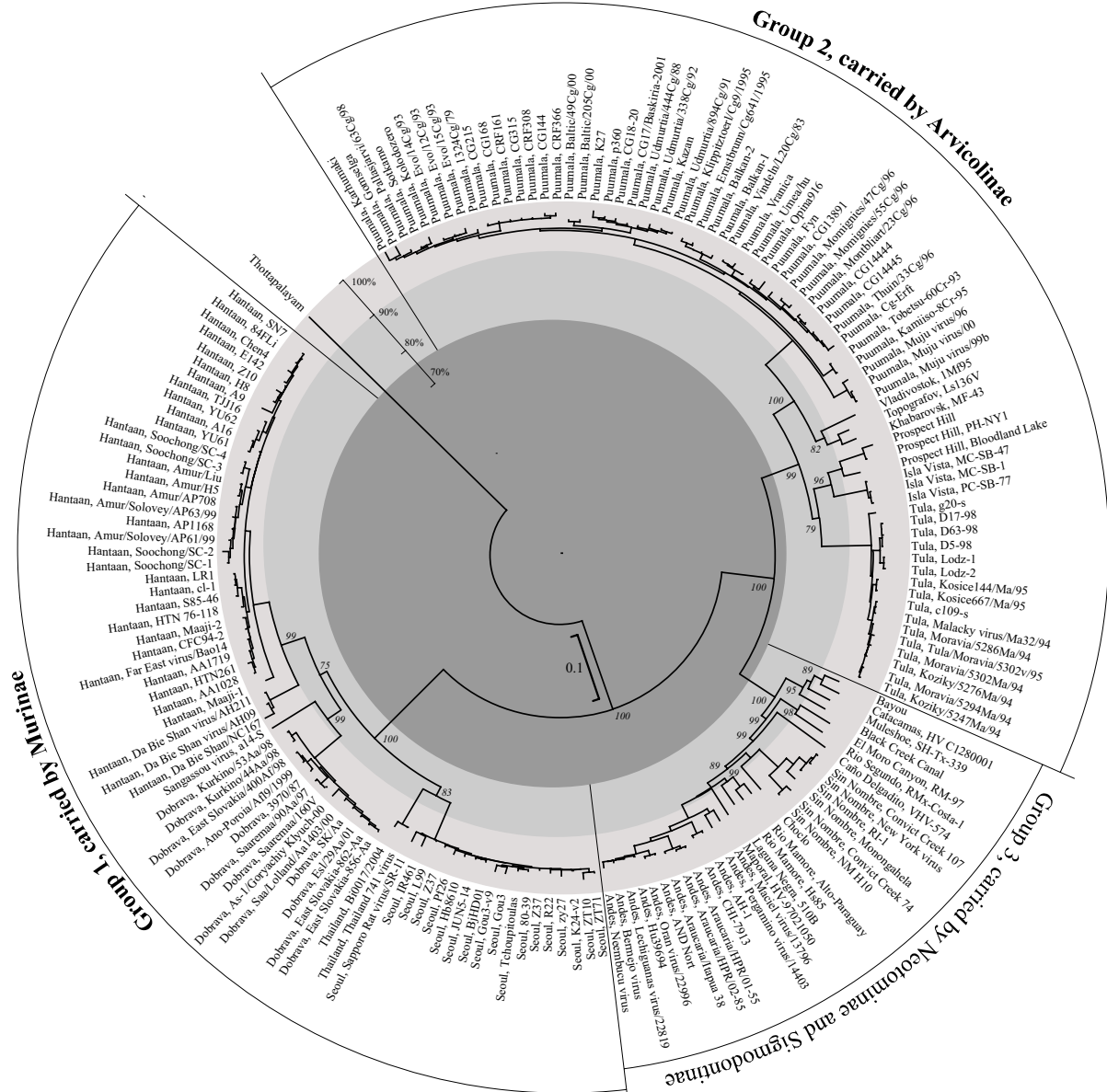
#### **4.2 Definition of hantavirus groups**

Our genetic approach led to an overall delineation of five distinct groups of hantaviruses, most of these already known and well-marked previously with more traditional serologic techniques. We assigned to hantavirus group 1 all hantaviruses carried by the Rodentia subfamily Murinae and belonging to the same Old World group as the prototype Hantaan virus. To hantavirus group 2 we assigned all New and Old World hantaviruses carried by the Rodentia subfamily Arvicolinae, with Puumala virus as its prototype. All New World hantaviruses, carried by the Rodentia subfamilies Neotominae and Sigmodontinae, were assigned to hantavirus group 3, with Sin Nombre virus as its prototype. Group 4 is represented by only one member, Thottapalayam virus, which is carried by a host animal of the order of Insectivora. Tentatively, a fifth group could be defined on the basis of a novel virus from Insectivora, Tanganya virus. At the moment however, only partial sequences are available for this novel virus.

#### **4.3 Definition of hantavirus species**

Using the above defined categories (*genogroup*, *species*, and *variant*) to classify hantaviruses, 18 hantavirus species could be identified (summarized in TABLE 2.1) spread over 5 hantavirus groups: Hantavirus group 1 with Dobrava-Belgrade virus, Hantaan virus, Sangassou virus, Seoul virus and Thailand virus; Hantavirus group 2 with Isla Vista virus, Prospect Hill virus, Puumala virus, Khabarovsk virus, Tula virus, and Vladivostok virus; Hantavirus group 3 with

Andes virus, Bayou virus, Caño Delgadito virus, Rio Segundo virus, and Sin Nombre virus; and Thottapalayam virus and Tanganya virus belonging to hantavirus group 4 and group 5 respectively.

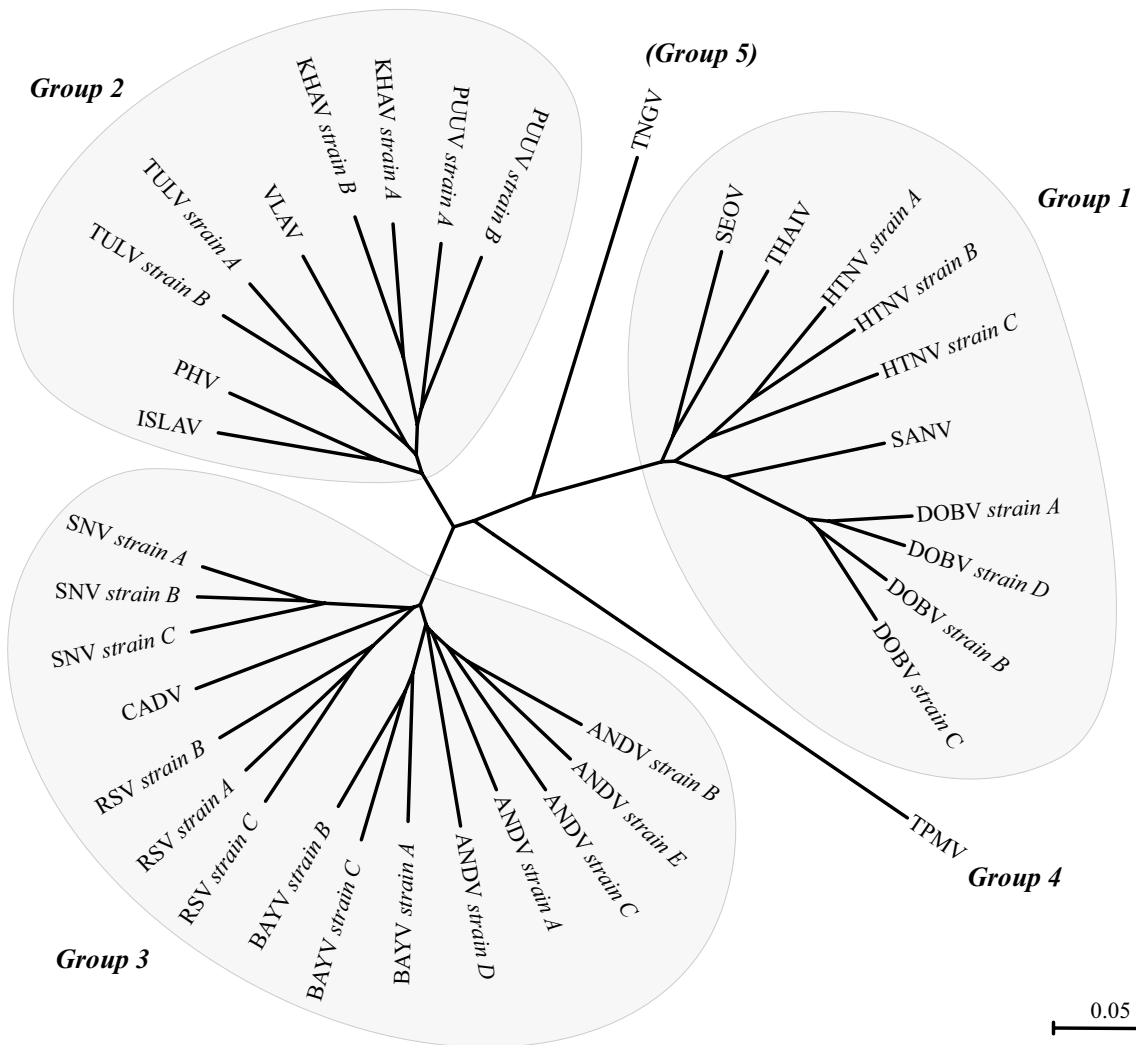


**FIGURE 2.4:** Circular neighbor-joining tree based on the complete amino acid sequences of the S segment, with indication of the similarity ranges: *genogroup* (up to 76% similarity, dark gray), *species* (76 to 90% similarity, medium gray) and *variant* (90 to 100% similarity, light gray). Only bootstrap values up to species level are shown. Accession numbers of the sequences used are summarized in TABLE 2.2.

#### 4.4 Definition of hantavirus strains

Maximum likelihood and neighbor-joining phylogenetic analysis of the S segment nucleotide and amino acid sequences respectively, revealed strains for several hantavirus species

(summarized in TABLE 2.1). Each of these strain branches had bootstrap values above 84%. No general consensus could be defined when using amino acid similarity analysis for strains only.



**FIGURE 2.5:** Maximum likelihood tree with 18 hantavirus species as defined by our proposed guidelines, divided into 5 groups. Group 1: DOBV, Dobrava-Belgrade virus; HTNV, Hantaan virus; SANGV, Sangassou virus; SEOV, Seoul virus; THAIV, Thailand virus. Group 2: ISLAV, Isla Vista virus; PHV, Prospect Hill virus; PUUV, Puumala virus; KHAV, Khabarovsk virus; TULV, Tula virus; VLAV, Vladivostok virus. Group 3: ANDV, Andes virus; BAYV, Bayou virus; CADV, Caño Delgadito virus; RSV, Rio Segundo virus; SNV, Sin Nombre virus. Group 4: TPMV, Thottapalayam virus. Group 5: TNGV, Tanganya virus.

For group 1, three strains could be identified within the Hantaan virus species. The first strains with the prototype Hantaan virus (strain 76-118) was assigned to Hantaan virus strain A, the second with Amur virus and Soochong virus was assigned to Hantaan virus strain B.

Da Bie Shan virus was assigned to Hantaan virus strain C. For Dobrava-Belgrade virus, maximum likelihood and neighbor-joining analysis revealed four well-supported strains in the Dobrava-Belgrade virus group: strain A with the viruses carried by *Apodemus flavicollis*, strain B with the viruses carried by *Apodemus agrarius*, strain C with Saaremaa virus, and strain D with *Apodemus ponticus* (FIGURE 2.5). For Puumala virus, a member of group 2, two strains were found by maximum likelihood and neighbor-joining analysis of the S segment. The strain containing the Puumala viruses carried by *Myodes glareolus* was assigned to Puumala virus strain A, whereas the Puumala virus strain containing Muju virus, isolated in Korea out of a *Eothenomys regulus*, was assigned to strain B. The Tula virus species was separated in strain A, containing the northern European strains, and strain B, containing the central European strains.

For the New World group 3 hantaviruses, five separate strains were found for the Andes virus species, three strains for Bayou virus and Rio Segundo virus, and three strains were found for the Sin Nombre virus species. Provisionally, we have divided the Andes virus cluster in five strains: strain A containing Araucaria virus, Bermejo virus, Lechiguanas virus, Maciel virus, Neembucu virus, Oran virus and Pergamino virus, strain B with Rio Mamore virus, strain C with Laguna Negra virus, strain D with Choclo virus, and strain E containing Maporal virus. Bayou virus was divided into strain A with Bayou virus, strain B with Black Creek Canal virus, and strain C with Muleshoe virus. Rio Segundo virus was separated into strain A containing Rio Segundo virus, strain B containing El Moro Canyon virus, and strain C containing Limestone Canyon virus. For Sin Nombre virus, the three strains could also be distinguished based on their S segment similarity ranges, and were assigned to as strain A, containing the Sin Nombre virus isolates, strain B, containing the New York virus isolates, and strain C, containing Monongahela virus.

#### **4.5 Determination of the minimum sequence length**

To determine the minimum length of S or M segment amino acid sequences needed for accurately identifying the correct hantavirus species, similarity plots and bootscanning analysis were performed on the complete amino acid M and S segment alignments. Every hantavirus sequence used in this study (TABLE 2.2) was analyzed using different frame lengths. Similarity plots revealed a minimum length of 300 aa for the S segment and a minimum length of 500 aa for the M segment, to have a difference respectively of 10 and 12%, between the query species and other hantavirus species, respectively.

**TABLE 2.1:** Summary of the species defined by using the new genetic classification rules. Hantavirus species are written in bold.

Group	Species	Strain	Members
1	<b><i>Dobrava-Belgrade virus</i></b>	A	Dobrava-Af <sup>(1)</sup>
		B	Dobrava-Aa <sup>(2)</sup>
		C	Saaremaa virus <sup>(2)</sup>
		D	Dobrava-Ap <sup>(4)</sup>
	<b><i>Hantaan virus</i></b>	A	Hantaan virus
		B	Amur virus , Soochong virus
		C	Da Bie Shan virus
	<b><i>Sangassou virus</i></b>		
	<b><i>Seoul virus</i></b>		
	<b><i>Thailand virus</i></b>		
2	<b><i>Isla Vista virus</i></b>		
	<b><i>Khabarovsk virus</i></b>	A	Khabarovsk virus
		B	Topografov virus
	<b><i>Prospect Hill virus</i></b>		
	<b><i>Puumala virus</i></b>	A	Puumala virus
		B	Muju virus
	<b><i>Tula virus</i></b>	A	Isolates from northern Europe
		B	Isolates from central Europe
<b><i>Vladivostok virus</i></b>			
3	<b><i>Andes virus</i></b>	A	Araucaria virus, Bermejo virus, Lechiguanas virus, Maciel virus, Neembucu virus, Oran virus, Pergamino virus
		B	Rio Mamore virus
		C	Laguna Negra virus
		D	Choclo virus
		E	Maporal virus
	<b><i>Bayou virus</i></b>	A	Bayou virus
		B	Black Creek Canal virus
		C	Muleshoe virus
	<b><i>Caño Delgadito virus</i></b>		
	<b><i>Rio Segundo virus</i></b>	A	Rio Segundo virus
		B	El Moro Canyon virus
		C	Limestone Canyon virus
	<b><i>Sin Nombre virus</i></b>	A	Sin Nombre virus
		B	New York virus
		C	Monongahela virus
4	<b><i>Thottapalayam virus</i></b>		
	(5) <b><i>Tanganya virus</i></b>		

- (1) Dobrava-Belgrade virus carried by *Apodemus flavicollis*  
(2) Dobrava-Belgrade virus carried by *Apodemus agrarius*  
(3) Dobrava-Belgrade virus carried by *Apodemus ponticus*

#### **4.6 Comparison with the ICTV defined hantavirus species**

Several hantaviruses, defined as distinct hantavirus species by ICTV, could not be identified with our newly defined criteria (more than 10% distance for S segment, and more than 12% distance for M segment) for the genetic classification of hantaviruses. Topografov virus has an amino acid similarity with Khabarovsk virus of 95.6% and 88.1% for the S and M segment respectively. New York virus shows high similarity with Sin Nombre virus on both the S and M segment amino acid sequences of 92.3 to 96.9% and 94.7 to 96.0% respectively. Bayou virus shows high S segment similarity with Black Creek Canal virus (92.4%) and Muleshoe virus (92.6%). Additionally, Black Creek Canal virus and Muleshoe virus have an S segment similarity of 90.3%. Rio Segundo virus and El Moro Canyon virus share an amino acid similarity of 91.1% in their S segment sequences.

During the process of analyzing all available sequences in Genbank, we came across sequences representing possible new hantavirus species, but which were not yet recognized as distinct species by ICTV. Sangassou virus has a similarity between 79.8 and 88.3% (S segment) in comparison to other members of group 1. Vladivostok virus [Genbank:AB011630] has the highest S segment similarity range of 88.9 to 89.4% with Khabarovsk virus of group 2. Tanganya virus, with only a short S segment sequence of 147 amino acids available in Genbank, has a similarity range of 21.5 to 53.3% with other hantavirus species.

Seven out of the 22 distinct hantavirus species as defined by ICTV, could not be delineated as distinct species with our criteria for the genetic classification of hantaviruses and were demarcated as strain. For each of them, the name of the first described virus was used to identify the species. Additionally, we have defined three new tentative hantavirus species, which gives us a total of 18 distinct hantavirus species.

#### **4.7 Multiple alignment of the hantavirus sequences**

Seven different alignment software programs were used to generate alignments of the different hantavirus segment sequences: AMAP v2.0 (for amino acid sequences only) (Schwartz and Pachter, 2007), ClustalW v1.83 (Thompson et al., 1994), MUSCLE v3.6, MAFFT v6.240 (Katoh et al., 2002), MAVID v2.0.4 (for nucleotide sequences only) (Bray and Pachter, 2004), M-Coffee (Moretti et al., 2007), and T-Coffee v5.05 (Notredame et al., 2000). These alignments were first corrected manually, after which the different alignments of one segment were compared with each other. No significant differences were noted between the alignments generated by the different programs, although great differences in

computational time needed to calculate these alignments was seen. The MUSCLE multiple alignment program v3.6 performed best with e.g. 19 seconds to calculate the S segment amino acid alignment, whereas ClustalW v1.83 needed more than 60 minutes to calculate an alignment from an identical amino acid sequence dataset. Moreover, similarity calculation by using the different alignments generated by the different software programs resulted in identical cut-off values for the definition of hantavirus groups and species.

#### ***4.8 Similarity and identity calculation methods***

After the definition of the cut-off values for hantavirus groups and species using the Poisson correction model, other substitution models were tested. For amino acid sequences the block substitution matrix (BLOSUM62) substitution model (Henikoff and Henikoff, 1992), the Jones-Taylor-Thornton (JTT) substitution model (Jones et al., 1992) and the percent accepted mutations (PAM-250) substitution model (Dayhoff et al., 1983) were tested supplementary. These substitution model resulted in different similarities, but the cut-off values for hantavirus groups and species remained the same.

## **5. DISCUSSION**

### ***5.1 Sequence-based classification of hantaviruses***

An exclusively genetic-based classification system is available for several viruses including Alphaviruses, Hepatitis C virus, noroviruses, papillomaviruses, Potyviridae, and rotaviruses (Gorziglia et al., 1990; Schuffenecker et al., 2001; de Villiers et al., 2004; Zheng et al., 2006). For hantaviruses, such classification system is not available. We hope to have convincingly argued that by using nucleotide sequences, no clear definition of hantavirus species can be put forth. Moreover, nucleotide differences do not always correlate with amino acid differences, which makes it highly questionable whether they are in any way relevant to the classification of the virus. We here propose that members of a specific hantavirus group must have an S segment amino acid distance (with Poisson correction) greater than 24.0% in comparison to members of other groups. Within each hantavirus group, similarity ranges can be further divided in *species* and *variant*, representing the distinction between different species in a given hantavirus group and the distinction between variants of a given species respectively. Thus we propose that different species should have an amino acid distance greater than 10.0%. Hantavirus strains can be distinguished clearly by using maximum likelihood and

neighbor-joining analysis. Sequences should have a minimum length of 300 aa (S segment), since shorter sequences might result in overlapping similarities between different hantavirus species, hampering proper identification of hantavirus species.

## **5.2 ‘Problematic’ hantavirus species**

With our defined guidelines for the genetic classification of hantaviruses, 18 distinct hantavirus species spread over 5 hantavirus groups could be defined (TABLE 2.1). This classification was compared with the current classification as defined by ICTV. Several ICTV hantavirus species could not be recognized based on our defined cut-off values.

### 5.2.1 Topografov virus

Khabarovsk virus was isolated from a *Microtus fortis* caught in the Khabarovsk region in far-eastern Russia (Horling et al., 1996), whereas Topografov virus was isolated from a *Lemmus sibiricus* caught near the Topografov river in the Taymyr Peninsula, Siberia, Russia (Plyusnin et al., 1996). Following our defined guidelines to genetically classify hantaviruses as presented above, these two viruses could not be distinguished as two distinct hantavirus species. Although these viruses are carried by two distinct arvicoline rodent species (Vapalahti et al., 1999), the viruses have a high similarity in their M segment and especially in their S segment amino acid sequences. Vapalahti and colleagues suggested a likely host switching event from the ancestor of *Myodes* which could have been the donor of an ancestral virus that became the common evolutionary origin of both Khabarovsk and Topografov virus (Vapalahti et al., 1999). Long deletions in the S and M segment 3’ noncoding regions of Khabarovsk virus compared with Topografov virus suggest that Topografov could represent a more ancestral virus than Khabarovsk virus (Vapalahti et al., 1999). Khabarovsk and Topografov virus are defined by ICTV as two separate species based on the fact that Khabarovsk and Topografov virus are carried by clearly distinct host species in which they are constantly maintained. However, these viruses cannot be distinguished using neutralization tests (Vapalahti et al., 1999). Only the first rule of the ICTV guidelines for the demarcation of hantavirus species is fulfilled, therefore we propose to assign Khabarovsk virus to a species of group 2, and Topografov virus as a strain of Khabarovsk virus.

### 5.2.2 New York virus

New York virus, which has been associated with typical HPS (Hjelle et al., 1995) was isolated from a *Peromyscus leucopus*, caught at an island off the coast of New York (Song et al.,

1994; Gavrilovskaya et al., 1999). This virus, demarcated as a separate hantavirus species by ICTV, shows high similarity with Sin Nombre virus on both the M and S segment amino acid sequences. Although New York virus can be distinguished undoubtedly by using neutralization tests (Gavrilovskaya et al., 1999), the virus could not be demarcated as a distinct hantavirus species using our criteria. The similarity range of New York virus does not meet the 10% similarity cutoff for the S segment and the 12% similarity cutoff for the M segment, as well as the ICTV criteria of a PID difference of 7% for both S and M segments, to be classified as a separate hantavirus species. As the virus is carried by a different, although closely related, rodent species than Sin Nombre virus, we propose to classify New York virus as a strain of Sin Nombre virus (Sin Nombre virus strain B).

### 5.2.3 South American hantaviruses

The hantavirus species endemic in South America are a group of closely related hantaviruses, some of them described only genotypically. Currently nine South American hantavirus species are demarcated by ICTV: Andes virus, Bayou virus, Black Creek Canal virus, Caño Delgadito virus, El Moro Canyon virus, Laguna Negra virus, Muleshoe virus, Rio Mamore virus, and Rio Segundo virus. However, analyzing the similarities between the different species revealed some discrepancies (FIGURE 2.1). Bayou virus has a high similarity with Black Creek Canal virus and Muleshoe virus. Moreover, Black Creek Canal virus and Muleshoe virus are carried by the same rodent species (*Sigmodon hispidus*), and until now, Muleshoe virus has no virus isolate available. Furthermore, no M segment sequences of Muleshoe virus are available in Genbank, what makes that this virus is in accordance with none of the ICTV guidelines for the demarcation of hantavirus species. Therefore, we propose to classify Bayou virus as a distinct hantavirus species in our genetic hantavirus classification with Black Creek Canal virus and Muleshoe virus as strain, strain B and strain C respectively. Rio Segundo virus has a similarity of 91.1% with El Moro Canyon virus and 90.5% with Limestone Canyon virus. El Moro Canyon virus has a similarity with Limestone Canyon virus of 88.5%. With our genetic classification, Rio Segundo virus was demarcated as a distinct species, with El Moro Canyon virus and Limestone Canyon virus as strain B and strain C of Rio Segundo virus respectively.

### **5.3 Tentative novel hantavirus species**

#### 5.3.1 Sangassou virus

Sangassou virus, has been recently isolated from a *Hylomyscus simus* or African Wood Mouse caught in Sangassou, Guinea. It was the first hantavirus to be described on the African continent (Klempa et al., 2006). Sangassou virus has a similarity between 79.8 and 88.3% (S segment) in comparison to other members of group 1. It is most closely related to Dobrava-Belgrade virus with a similarity range between 87.1 and 88.3%. Sangassou virus can be undoubtedly classified as a new hantavirus species of group 1.

#### 5.3.2 Tanganya virus

Tanganya virus is only very recently discovered in a *Crocidura theresae* in Guinea, Africa (Klempa et al., 2007). Although there is only a short S segment sequence of 147 amino acids available [Genbank:EF050555], similarity analysis revealed that this virus is convincingly distinct from other hantavirus species. Therefore, Tanganya virus is provisionally assigned to a fifth distinct hantavirus group.

#### 5.3.3 Vladivostok virus

Vladivostok virus, a hantavirus isolated out of a *Microtus fortis* or Reed vole, has a partial S segment sequence available of 403 amino acids in Genbank, with a similarity range of the S segment between 79.5 and 89.4% in comparison to members of group 2. Vladivostok virus has the highest similarity for the S segment with Khabarovsk virus, but is still in complete accordance with our defined rules for genetically classification of hantaviruses. Vladivostok virus is assigned to group 2 as a new hantavirus species.

### **5.4 Conclusions**

Virus classification places viruses in a series of classes or taxonomic categories with a hierarchical structure. These classes are abstractions, i.e. conceptual constructions produced by the human mind (van Regenmortel and Mahy, 2004). A virus species, in this particular case a hantavirus species, is defined by ICTV as “a polythetic class of viruses that constitute a replicating lineage and occupy a particular ecological niche e.g. a rodent reservoir species or subspecies” (van Regenmortel, 1990; Nichol et al., 2005). A polythetic class consists of members which have a number of properties in common but which do not all share a single common property that could be used as a defining and distinguishing feature of the species because it is absent in other species (van Regenmortel and Mahy, 2004). Currently,

hantaviruses are demarcated into species based on 4 criteria (Nichol et al., 2005). Although these specific characteristics are essential for demarcating individual hantavirus species and for the construction of an acceptable classification system, identifying a hantavirus isolate as a member of a certain hantavirus species by considering only a few properties may be possible. Absolute care should be taken with the introduction of new hantavirus species. Since virus species are polythetic, the comparison should involve a number of different characteristics rather than the presence or absence of a single key feature. In recent years, several publications have proposed the demarcation of new hantavirus species based on a single difference like the difference in the natural host rodent species, or the absence of related human disease, without taking notice of the ICTV species demarcation guidelines. Deciding whether an individual virus isolate represents a new hantavirus species can not be judged by a single parameter difference. Unfortunately, several hantavirus isolates demarcated as distinct species by ICTV, do not meet the criteria defined by these ICTV guidelines, but are demarcated as distinct hantavirus species based on a single parameter difference. This may often lead to uncertainty about which rules to use for hantavirus species demarcation. We showed that only amino acid sequences (with Poisson correction), and not nucleotide sequences allowed efficient demarcation between different hantavirus species, and proposed new criteria for demarcation of hantavirus groups (amino acid distance >24.0%) and species (amino acid distance >10.0%). Hantavirus strains were defined using well supported branches (bootstrap values higher than 84%) with maximum likelihood and neighbor-joining analysis. This approach resulted in confirming most of the previously defined hantavirus species, adding three new, very recently described, species (Sangassou virus, Tanganya virus, and Vladivostok virus), but seven ICTV species could not be identified as such and were demarcated as strains of other hantavirus species.

The most important advantage of the proposed classification scheme is that it is in almost perfect agreement with the current ICTV classification and that it is very easy to perform and therefore also easy to prove. Discrepancies to the current ICTV classification were found only in case of those viruses which were classified as distinct species notwithstanding the fact that they did not fulfill all the ICTV species demarcating criteria.

In summary, we propose to adjust the second rule of the ICTV classification guidelines (“*a 7% difference in amino acid identity when comparing the complete S segment and M segment sequences*”) to a more appropriate rule, “*a 10% difference in S segment similarity or a 12% difference in M segment similarity based on complete amino acid sequences*” in accordance with the current situation in the hantavirus field. In addition, this proposed guideline can be

used to genetically identify tentative hantavirus species in awaiting of the more difficult to perform neutralization tests. For hantaviruses found without a virus isolate, these guidelines can form an alternative, scientific based approach in the identification of the correct hantavirus species.

**TABLE 2.2:** Complete S segment sequences used for the genetic classification of hantaviruses. The listing is alphabetical and does not reflect phylogenetic relationships. Hantavirus species with a virus isolate are written in bold.

<i>Virus species name</i> (abbreviation)	Variant	Genbank accession	Source and Host name
<b><i>Andes virus</i><sup>(1)</sup></b> <b>(ANDV)</b>	AH-1	AF324902	Oligoryzomys longicaudatus Long-tailed Pygmy Rice Rat (Colilargo)
	AND Nort	AF325966	Oligoryzomys chacoensis Chacoan Pygmy Rice Rat (Colilargo)
	Anajatuba virus/Of58	DQ451829 <sup>(2)</sup>	Oligoryzomys fornesi Fornes' Pygmy Rice Rat (Colilargo)
	Araucaria virus/Itapua 37	DQ345765	Oligoryzomys nigripes Black-footed Pygmy Rice Rat (Colilargo)
	Araucaria virus/Itapua 38	DQ345766	Oligoryzomys nigripes Black-footed Pygmy Rice Rat (Colilargo)
	Araucaria virus/HPR/01-55	AY740623	Homo sapiens Human
	Araucaria virus/HPR/02-71	AY740624	Homo sapiens <i>Human</i>
	Araucaria virus/HPR/02-72	AY740625	Homo sapiens <i>Human</i>
	Araucaria virus/HPR/02-73	AY740626	Homo sapiens <i>Human</i>
	Araucaria virus/HPR/02-85	AY740627	Homo sapiens <i>Human</i>
	Araucaria virus/HPR/03-95	AY740628	Homo sapiens <i>Human</i>
	Araucaria virus/HPR/03-97	AY740629	Homo sapiens <i>Human</i>
	Araucaria virus/HPR/03-99	AY740630	Homo sapiens <i>Human</i>
	Araucaria virus/HPR/04-102	AY740633	Homo sapiens <i>Human</i>
	Bermejo virus	AF482713	Oligoryzomys chacoensis Chacoan Pygmy Rice Rat (Colilargo)
	CHI-7913	AY228237	Homo sapiens Human
	Chile-9717869	NC_003466	Oligoryzomys longicaudatus Long-tailed Pygmy Rice Rat (Colilargo)
	Choclo virus	DQ285046	Oligoryzomys fulvescens Fulvous Pygmy Rice Rat (Colilargo)
	Hu39694	AF482711	Homo sapiens Human
	Lechiguanas virus/22819	AF482714	Oligoryzomys flavescens Yellow Pygmy Rice Rat (Colilargo)
	Maciel virus/13796	AF482716	Necromys benefactus Argentine Akodont or Dark Field Mouse
	Maporal virus/HV-97021050	AY267347	Oligoryzomys fulvescens Fulvous Pygmy Rice Rat (Colilargo)

	Neembucu virus	DQ345763	Oligoryzomys chacoensis Chacoan Pygmy Rice Rat (Colilargo)
	Oran virus/22996	AF482715	Oligoryzomys longicaudatus Long-tailed Pygmy Rice Rat (Colilargo)
	P5/Cajuru	EF571895	Homo Sapiens Human
	Paranoa	EF57661	Homo sapiens Human
	Pergamino virus/14403	AF482717	Akodon azarae Azara's Akodont or Azara's Grass Mouse
<b>Bayou virus<sup>(1)</sup> (BAYV)</b>	Bayou virus	L36929	Homo sapiens; host: Oryzomys palustris Human; Marsh Rice Rat
	Catacamas virus/HV C1280001	DQ256126	Oryzomys couesi <i>Coues' Rice Rat</i>
<b>Black Creek Canal virus<sup>(1)</sup> (BCCV)</b>		L39949	Sigmodon hispidus <i>Hispid Cotton Rat</i>
<b>Caño Delgadito virus<sup>(1)</sup> (CADV)</b>	VHV-574	DQ285566	Sigmodon alstoni Alston's Rice Rat
<b>Dobrava-Belgrade virus<sup>(1)</sup> (DOBV)</b>	3970/87	L41916	Apodemus flavicollis Yellow-necked Field Mouse
	Ano-Poroia/13Af/99	AJ410619	Apodemus flavicollis Yellow-necked Field Mouse
	Ano-Poroia/Af19/1999	AJ410615	Apodemus flavicollis Yellow-necked Field Mouse
	Ano-Poroia/Af19/1999	NC_005233	Apodemus flavicollis Yellow-necked Field Mouse
	As-1/Goryachiy Klyuch-2000	AF442622	Apodemus ponticus Caucasus Field Mouse
	East Slovakia/400Af/98	AY168576	Apodemus flavicollis Yellow-necked Field Mouse
	East Slovakia-856-Aa	AJ269549	Apodemus agrarius Striped Field Mouse
	East Slovakia-862-Aa	AJ269550	Apodemus agrarius Striped Field Mouse
	Esl/29Aa/01	AY533118	Apodemus agrarius Striped Field Mouse
	Esl/34Aa/01	AY961618	Apodemus agrarius Striped Field Mouse
	Esl/81Aa/01	AY533120	Apodemus agrarius Striped Field Mouse
	Kurkino/44Aa/98	AJ131672	Apodemus agrarius Striped Field Mouse
	Kurkino/53Aa/98	AJ131673	Apodemus agrarius Striped Field Mouse
	P-s1223/Krasnodar-2000	AF442623 <sup>(2)</sup>	Homo sapiens Human
	Saaremaa/160V	AJ009773	Apodemus agrarius Striped Field Mouse
	Saaremaa/90Aa/97	AJ009775	Apodemus agrarius Striped Field Mouse
	Saaremaa/Lolland/Aa1403/2000	AJ616854	Apodemus agrarius Striped Field Mouse
	SK/Aa	AY961615	Apodemus agrarius Striped Field Mouse

<i>El Moro Canyon virus</i> <sup>(1)</sup> (ELMCV)	RM-97	U11427	Reithrodontomys megalotis Western Harvest Mouse
<i>Hantaan virus</i> <sup>(1)</sup> (HTNV)	84FLi	AF366568	Homo sapiens Human
	A16	AF288646	Apodemus agrarius Striped Field Mouse
	A9	AF329390	Apodemus agrarius Striped Field Mouse
	AA1028	AF427318	Apodemus agrarius Striped Field Mouse
	AA1719	AF427319	Apodemus agrarius Striped Field Mouse
	AA2499	AF427320	Apodemus agrarius Striped Field Mouse
	AH09	AF285264	<i>n.g.</i> <sup>(3)</sup>
	AH211	AF288647	<i>n.g.</i>
	Amur virus/AP708	AF427322	Apodemus peninsulae Korean Field Mouse
	Amur virus/AP1371	AF427324	Apodemus peninsulae Korean Field Mouse
	Amur virus/B78	AB127997	Homo sapiens Human
	Amur virus/H5	AB127996	Homo sapiens Human
	Amur virus/Liu	AF288649	Homo sapiens Human
	Amur virus/Solovey/AP61/1999	AB071183	Apodemus peninsulae Korean Field Mouse
	Amur virus/Solovey/AP63/1999	AB071184	Apodemus peninsulae Korean Field Mouse
	AP1168	AF427323	Apodemus agrarius Striped Field Mouse
	CJAp93	EF208929	Apodemus peninsulae Korean Field Mouse
	CFC94-2	X95077	Homo sapiens Human
	Chen4	AB027101	Homo sapiens Human
	cl-1	D25530	derived from HTNV 76-118
	cl-2	D25533	derived from HTNV 76-118
	CUMC-B11	U37768	<i>n.g.</i>
	E142	AF288644	<i>n.g.</i>
	Far East virus/Bao14	AB127998	Apodemus agrarius Striped Field Mouse
	H8	DQ658415	Homo sapiens Human
	HTN261	AF252259	<i>n.g.</i>
	HTN 76-118	NC_005218	Apodemus agrarius Striped Field Mouse
HTN 76-118 <sup>(4)</sup>	M14626	Apodemus agrarius Striped Field Mouse	
LR1	AF288294	<i>n.g.</i>	

	Maaji-1	AF321094	<i>n.g.</i>
	Maaji-2	AF321095	<i>n.g.</i>
	NC167	AB027523	Niviventer confucianus Chinese White-bellied Rat
	RG9	AF288296	<i>n.g.</i>
	S85-46	AF288659	<i>n.g.</i>
	SN7	AF288657	<i>Crocidura russula</i> <sup>(5)</sup> Greater White-toothed Shrew
	Soochong virus-1/SC-1	AY675349	<i>Apodemus peninsulae</i> Korean Field Mouse
	Soochong virus-1/SC-2	AY675350	<i>Apodemus peninsulae</i> Korean Field Mouse
	Soochong virus-1/SC-3	AY675351	<i>Apodemus peninsulae</i> Korean Field Mouse
	Soochong virus-1/SC-4	AY675352	<i>Apodemus peninsulae</i> Korean Field Mouse
	TJJ16	AY839871	<i>n.g.</i>
	YU61	AY748308	<i>n.g.</i>
	YU62	AY748309	<i>n.g.</i>
	Z10 (vaccine strain)	NC_006433	Homo sapiens Human
	Z251	EF595840	<i>n.g.</i>
<b><i>Isla Vista virus</i><sup>(1)</sup> (ISLAV)</b>	MC-SB-1	U31534	Microtus californicus California Vole
	MC-SB-47	U19302	Microtus californicus California Vole
	PC-SB-77	U31535	Peromyscus californicus California Deermouse
<b><i>Khabarovsk virus</i><sup>(1)</sup> (KHAV)</b>	Khabarovsk virus/MF-43	U35255	Microtus fortis Reed Vole
<b><i>Laguna Negra virus</i><sup>(1)</sup> (LANV)</b>	510B	AF005727	Calomys laucha Vesper Mouse or White Paunch Mouse
<b><i>Muleshoe virus</i><sup>(1)</sup> (MULV)</b>	SH-Tx-339	U54575	Sigmodon hispidus Hispid Cotton Rat
<b><i>New York virus</i><sup>(1)</sup> (NYV)</b>	RI-1	U09488	Homo Sapiens Human
<b><i>Prospect Hill virus</i><sup>(1)</sup> (PHV)</b>	Bloodland Lake virus	U19303	Microtus ochrogaster Prairie Vole
	PH-NY1	U47136	<i>n.g.</i>
	Prospect Hill virus	M34011	Microtus pennsylvanicus Meadow Vole
	Prospect Hill virus	X55128	Microtus pennsylvanicus Meadow Vole

<i>Puumala virus</i> <sup>(1)</sup> ( <i>PUUV</i> )	1324Cg/79	Z46942	Myodes glareolus Bank Vole
	Balkan-1	AJ314600	Myodes glareolus Bank Vole
	Balkan-2	AJ314601	Myodes glareolus Bank Vole
	Baltic/205Cg/00	AJ314599	Myodes glareolus Bank Vole
	Baltic/49Cg/00	AJ314598	Myodes glareolus Bank Vole
	CG 13891	U22423	Myodes glareolus Bank Vole
	CG144	AF367064	Myodes glareolus Bank Vole
	CG14444	AJ277075	Myodes glareolus Bank Vole
	CG14445	AJ277076	Myodes glareolus Bank Vole
	CG168	AF367065	Myodes glareolus Bank Vole
	CG17/Baskiria-2001	AF442613	Myodes glareolus Bank Vole
	CG18-20	M32750	Myodes glareolus Bank Vole
	CG215	AF367066	Myodes glareolus Bank Vole
	CG222	AF367067	Myodes glareolus Bank Vole
	CG315	AF367068	Myodes glareolus Bank Vole
	Cg-Erft	AJ238779	Myodes glareolus Bank Vole
	Couvin/59Cg/97	AJ277034	Myodes glareolus Bank Vole
	CRF161	AF367069	Myodes rufocanus Gray Red-Backed Vole
	CRF308	AF367070	Myodes rufocanus Gray Red-backed Vole
	CRF366	AF367071	Myodes rufocanus Gray Red-Backed Vole
	Ernstbrunn/Cg641/1995	AJ888752	Myodes glareolus Bank Vole
	Evo/12Cg/93	Z30702	Myodes glareolus Bank Vole
	Evo/13Cg/93	Z30703	Myodes glareolus Bank Vole
	Evo/14Cg/93	Z30704	Myodes glareolus Bank Vole
	Evo/15Cg/93	Z30705	Myodes glareolus Bank Vole
	Fusong 114-05	EF488804	<i>n.g.</i>
	Fusong 119-05	EF488803	<i>n.g.</i>
	Fusong 843-06	EF488805	<i>n.g.</i>
	Fusong 900-06	EF488806	<i>n.g.</i>
	Fusong-Cr-247	EF442087	Myodes rufocanus Gray Red-backed Vole

Fusong-Cr-275	EF442091	Myodes rufocanus Gray Red-backed Vole
Fyn	AJ238791	Myodes glareolus Bank Vole
Gomselga	AJ238790	Myodes glareolus Bank Vole
K27	L08804	Myodes glareolus Bank Vole
Kamiiso-8Cr-95 (Hokkaido virus)	AB010730	Myodes rufocanus Gray Red-backed Vole
Karhumaki	AJ238788	Myodes glareolus Bank Vole
Kazan	Z84204	<i>n.g.</i>
Klippitztoerl/Cg9/1995	AJ888751	Myodes glareolus Bank Vole
Kolodozero	AJ238789	Myodes glareolus Bank Vole
Momignies/47Cg/96	AJ277032	Myodes glareolus Bank Vole
Momignies/55Cg/96	AJ277033	Myodes glareolus Bank Vole
Montbliart/23Cg/96	AJ277031	Myodes glareolus Bank Vole
Muju virus/00	DQ138128	<i>Eothenomys regulus</i> Royal Vole
Muju virus/96	DQ138133	<i>Eothenomys regulus</i> Royal Vole
Muju virus/99a	DQ138140	<i>Eothenomys regulus</i> Royal Vole
Muju virus/99b	DQ138142	<i>Eothenomys regulus</i> Royal Vole
Opina916	AF294652	Myodes glareolus Bank Vole
p360	L11347	<i>n.g.</i>
Pallasjarvi/63Cg/98	AJ314597	Myodes glareolus Bank Vole
Sotkamo	X61035	Myodes glareolus Bank Vole
Sotkamo	NC_005224	Myodes glareolus Bank Vole
Thuin/33Cg/96	AJ277030	Myodes glareolus Bank Vole
Tobetsu-60Cr-93	AB010731	Myodes rufocanus Gray Red-Backed Vole
Udmurtia/338Cg/92	Z30708	Myodes glareolus Bank Vole
Udmurtia/444Cg/88	Z30706	Myodes glareolus Bank Vole
Udmurtia/458Cg/88	Z30707	Myodes glareolus Bank Vole
Udmurtia/894Cg/91	Z21497	Myodes glareolus Bank Vole
Umea/hu	AY526219	Homo Sapiens Human
Vindeln/L20Cg/83	Z48586	Myodes glareolus Bank Vole
Vranica	U14137	Myodes glareolus Bank Vole

<b>Rio Mamore virus</b> <sup>(1)</sup> (RIOMV)	OM-556	U52136 <sup>(2)</sup>	Oligoryzomys microtis Small-eared Pygmy Rice Rat
	Alto-Paraguay virus	DQ345762	Holochilus chacarius <i>Chacoan Marsh Rat</i>
	Rio Mearim virus/Hs85	DQ451828 <sup>(2)</sup>	Holochilus sciureus Amazonian Marsh Rat
<b>Rio Segundo virus</b> <sup>(1)</sup> (RIOSV)	Limestone Canyon virus/68273	AF307322 <sup>(2)</sup>	Peromyscus boylii Brush Deermouse
	RMx-Costa-1	U18100	<i>Reithrodontomys mexicanus</i> Mexican Harvest Mouse
<b>Sangassou virus</b> (SANGV)	SA-14	DQ268650 <sup>(2)</sup>	Hylomyscus simus African Wood Mouse
<b>Seoul virus</b> <sup>(1)</sup> (SEOV)	80-39	NC_005236	Rattus norvegicus Brown Rat or Norway Rat
	BjHD01	AY627049	<i>n.g.</i>
	Gou3	AB027522	Rattus rattus Black Rat
	Gou3-v9 (Vero E6 adapted)	AF288651	Rattus rattus Black Rat
	Hb8610	AF288643	<i>n.g.</i>
	IR461	AF329388	Homo sapiens Human
	JUN5-14	DQ217791	Rattus norvegicus Brown Rat or Norway Rat
	K24-e7 (Vero E6 adapted)	AF288653	<i>n.g.</i>
	K24-v2 (Vero E6 adapted)	AF288655	<i>n.g.</i>
	L99	AF488708	Homo sapiens Human
	Pf26	AY006465	<i>n.g.</i>
	R22	AF488707	Homo sapiens Human
	Sapporo Rat virus/SR-11	M34881	Rattus norvegicus Brown Rat or Norway Rat
	Tchoupitoulas virus	AF329389	<i>n.g.</i>
	YZG – Changchun	EF536376	Rattus norvegicus albus Brown Rat or Norway Rat (albino)
	Z37	AY605933	<i>n.g.</i>
	Z37	NC_006434	<i>n.g.</i>
	ZT10	AY766368	<i>n.g.</i>
	ZT71	AY750171	<i>n.g.</i>
	zy27	AF406965	<i>n.g.</i>

<b><i>Sin Nombre virus</i><sup>(1)</sup></b> (SNV)	Convict Creek 74 virus	L33816	Peromyscus maniculatus North American Deermouse
	Convict Creek 107 virus	U47135	Homo sapiens Human
	New York	U29210	Peromyscus leucopus White-Footed Deermouse
	NM H10	NC_005216	Peromyscus maniculatus North American Deermouse
	NM H10	L25784	Peromyscus maniculatus North American Deermouse
	NM R11	L37904	Peromyscus maniculatus North American Deermouse
	Monongahela virus	U32591	Peromyscus maniculatus nubiterrae Cloudland Deermouse
<b><i>Tanganya virus</i></b> (TGNV)	Tan826	EF050455 <sup>(2)</sup>	Crocidura theresae Therese's Shrew
<b><i>Thailand virus</i><sup>(1)</sup></b> (THAIV)	Thailand 741 virus	AB186420	Bandicota indica Greater Bandicoot Rat
	Nakhon Ratchasima/Bi0017/2004	AM397664	Bandicota indica Greater Bandicoot Rat
<b><i>Thottapalayam virus</i><sup>(1)</sup></b> (TPMV)	807039	AY526097	<i>Suncus murinus</i> <sup>(6)</sup> Asian House Shrew
<b><i>Topografov virus</i><sup>(1)</sup></b> (TOPV)	Ls136V	AJ011646	Lemmus sibiricus <i>Siberian Brown Lemming</i>
<b><i>Tula virus</i><sup>(1)</sup></b> (TULV)	c109-s	AF164094	Microtus arvalis Common Vole
	D17-98	AF289820	Microtus arvalis Common Vole
	D5-98	AF289819	Microtus arvalis Common Vole
	D63-98	AF289821	Microtus arvalis Common Vole
	g20-s	AF164093	Microtus arvalis Common Vole
	Kosice144/Ma/95	Y13979	Microtus arvalis Common Vole
	Kosice667/Ma/95	Y13980	Microtus arvalis Common Vole
	Koziky/5247Ma/94	AJ223600	Microtus arvalis Common Vole
	Koziky/5276Ma/94	AJ223601	Microtus arvalis Common Vole
	Lodz-1	AF063892	Microtus arvalis Common Vole
	Lodz-2	AF063897	Microtus arvalis Common Vole
	Malacky virus/Ma32/94	Z48235	Microtus arvalis Common Vole
	Malacky virus/Ma370/94	Z68191	Microtus arvalis Common Vole
	Moravia/5286Ma/94	Z48573	Microtus arvalis Common Vole
	Moravia/5293Ma/94	Z48574	Microtus arvalis Common Vole
	Moravia/5294Ma/94	Z48741	Microtus arvalis

	Moravia/5302Ma/94	Z49915	Common Vole Microtus arvalis
	Moravia/5302v/95	NC_005227	Common Vole Microtus arvalis
	Tula/Moravia/5302v/95	Z69991	Common Vole Microtus arvalis & rossiaemeridionalis Common Vole & Sibling Vole
<b>Vladivostok virus (VLAV)</b>	Vladivostok 1Mf95	AB011630 <sup>(2)</sup>	Microtus fortis Reed Vole

(1) Hantavirus species as defined by the latest report of ICTV (Nichol et al., 2005)

(2) partial sequence, no complete S segment sequence available in Genbank

(3) *n.g.*: not given

(4) sequence is equal to Genbank accession number NC\_005218

(5) *Crocidura russula* or Greater White-toothed Shrew, Order Soricomorpha, family Soricidae, subfamily Crocidurinae

(6) *Suncus murinus* or Asian House Shrew, Order Soricomorpha, family Soricidae, subfamily Crocidurinae

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## **GETHA: A WEB-BASED GENOTYPING TOOL FOR HANTAVIRUSES**

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This chapter is submitted to:

**Piet Maes, Jan Clement and Marc Van Ranst.** 2007. GeTHa: a web-based genotyping tool for hantaviruses. *BMC Bioinformatics* submitted.

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## 1. SUMMARY

Hantaviruses, members of the family Bunyaviridae, are classically divided into different species using serological methods. However, an increasing number of hantaviruses are being detected and classified by using exclusively molecular techniques and phylogenetic analyses, thus without a proper virus isolate. Several phylogenetic methods are available, but most often they are arduous and complex. We here describe an easy-to-use automated web-based tool for the genetic classification of hantavirus species by using amino acid sequences from the S segment.

The GeTHa web-based application (Genotyping Tool for Hantaviruses) was developed using Java and PERL scripts, and combines phylogenetic analyses with similarity comparison for the classification of hantaviruses. The analyses of previously well-characterized hantavirus sequences found in Genbank showed that all tested sequences could be correctly classified when using the GeTHa web application.

The GeTHa tool helps users classify their hantavirus sequences. This tool is platform independent and is assessable by Linux, Mac and Windows users. The hantavirus genotyping tool can be accessed freely at <http://fons.med.kuleuven.be/getha2>.

## 2. INTRODUCTION

Serological and phylogenetic analysis of the hantavirus species revealed five groups of hantaviruses, each carried by a different Rodentia or Soricomorpha subfamily (Carey et al., 1971; Schmaljohn and Hjelle, 1997; Kruger et al., 2001; Klempa et al., 2007). The current classification of hantaviruses (Nichol et al., 2005) has 22 defined species, principally based on serological features of the different viruses. More often, these viruses are discovered by using molecular techniques, thus without a virus isolate, and classified by applying phylogenetic methods. Until recently, no generally accepted strategy was available regarding the genetic classification of hantaviruses. Some basic guidelines related to this topic are described in Chapter 2. By applying these guidelines, hantaviruses can be classified into five distinct hantavirus groups based on pairwise similarities: group 1 carried by the *Rodentiae* subfamily *Murinae*, group 2 carried by the *Rodentiae* subfamily *Arvicolinae*, group 3 carried by the *Rodentiae* subfamilies *Neotominae* and *Sigmodontinae*, and group 4 and group 5 with Thottapalayam virus and Tanganya virus respectively as their only members. Hantavirus group demarcation is based on S segment similarity lower than 76.0% (Poisson correction

model), and can be clearly distinguished in neighbor-joining phylogenetic dendograms. Different hantavirus species belonging to the same genogroup have S segment similarities above 76.0%. Pairwise similarities above 90.0% correspond to different variants of the same hantavirus species. Strain demarcation within a hantavirus species can be done using neighbor-joining analyses. Although all three segments can theoretically be used to genetically classify hantaviruses, it has been recommended to use amino acid sequences from the S segment.

To assist researchers in the genetic classification of hantaviruses, we have developed a web-based algorithm using phylogenetic methods and the criteria described above. The tool was developed in Java and can be easily accessed at <http://fons.med.kuleuven.be/getha2>.

### **3. IMPLEMENTATION**

#### **3.1 *S* segment reference sequences**

The high number of complete S segment sequences available in Genbank (prior to June 2007) makes the S segment the pre-eminent hantavirus segment to use as a basis for a genetic classification. We have chosen not to include the option to classify a hantavirus based on its M or L segment because there are too few complete sequences of these segments available to perform an accurate classification.

As reference sequences, 185 sequences were selected out of 218 complete S segment sequences available in Genbank (Benson et al., 2005). These 218 sequences were first translated to amino acid sequences and were checked for identical amino acid sequences using DAMBE v4.2.13 software (Xia and Xie, 2001) which gave 185 unique and complete reference S segment sequences. The amino acid sequences of these 185 reference strains were aligned using ClustalW (Thompson et al., 1994). The alignment was formatted using the multiple sequence alignment editor GeneDoc v2.6.002, and similarities were calculated with Mega v3.1 software (Kumar et al., 2004) by using the Poisson correction model. The correct species of these reference strains was determined by using the cut-off values for genogroup (more than 76.0% similarity) and species (more than 90.0% similarity). Where appropriate, strains were determined by using neighbor-joining method with bootstrap analysis based on 10000 replicates to estimate the statistical support for the branching pattern using Mega v3.1 software.

### ***3.2 Web application procedure***

In an initial step, the user can enter a nucleotide or amino acid sequence in FASTA format. A nucleotide sequence is translated to amino acids by first identifying the coding open reading frame by comparison with a small number of reference strains of the S, M and L segments. In this step, the amino acid query is identified as an S, M or L segment sequence. This part was included in the web application to be able to include without difficulty, classification based on M and L segment sequences in the future.

After this initial step, a new alignment is created based on the query sequence and the reference strain alignment of 185 S segment amino acid sequences by using the profile alignment functions of ClustalW. This new alignment is used to create an uncorrected similarity matrix. In a next step, the similarity matrix is analyzed and the corresponding genogroup and species are identified by using the previously described rules for the genetic classification of hantaviruses using S segment sequences (Chapter 2). Briefly, an amino acid query sequence is a member of a specific genogroup if its (uncorrected) similarity range with other members of the genogroup is above 76.0%. A new genogroup can be defined if the similarity range of the query sequence is between 30.0% and 76.0%. Query sequences with a similarity range lower than 30.0% are considered not to be hantaviruses. A new hantavirus species is defined when its similarity range is between 76.0% and 90.0%, and a query sequence can be considered as an already defined hantavirus species when its similarity is above 90.0% with an existing species.

After the analysis of the similarity matrix, a neighbor-joining phylogenetic tree with 100 bootstrap replicates, is constructed by using the Paup\* v4.0b10 software to validate the results obtained with the similarity analysis. If the query sequence forms a well-supported monophyletic cluster (bootstrap >70%) with other sequences of a hantavirus species and if the similarity range with these sequences is above 90.0%, it can be concluded that the query sequence is a part of that specific hantavirus species. If the query sequence forms a well-supported monophyletic branch, with a similarity range below 90.0%, it can be concluded that this sequence represents a new hantavirus species. New genogroups are defined as monophyletic branches (bootstrap >70%) with a similarity range between 30.0% and 76.0%. In the same way partial S segment query sequences are scored during this analysis. It has been shown that partial sequences below 300 amino acids in length can not always be used for an accurate species classification (Chapter 2). Therefore, the program makes a conclusion only if the query sequence is 300 amino acids or more. For shorter sequences, a preliminary conclusion is made only if a well-supported branch (bootstrap >70%) is found in the

neighbor-joining tree. Query sequences with lengths below 150 amino acids, are not considered in the GeTHa tool. Results obtained using these sequences are not trustworthy.

**Results** (analysis 7009)

**Query sequence name:** AB297665 PUUV strain DTK/Ufa-97

**Origin of query sequence:** S segment (nucleocapsid protein)

**Amino acid sequence used for the analysis:** (438 aa)  
 TRTTGMSDLTDIQEEITRHEQQLVVARQKLDKAERAVEVYPDDVNKNTLQARQQTVSALEDKLADYKRRMADAVSRKKMD  
 TKPTDPTGIEPDDHLKERSRLRYGNVLDVNAIDIEEPSGQTADWYTIQVYVIGFTIPIILKALYMLSTRGRQTVKENKGT  
 RIRFKDDTSFEDINGIRRPKHLVSMPTAQSTMKAEELTPGRFRTIVCGLFPTQIQVRNIMSPVMGVIGFSFFVKDWPEK  
 IREFMEKECPFIKPEVKPGTPAQEVEFLKRNRYFMTRQDVLKDNHVADIDKLIDYAASGDPTSPDDIESPNAPWVFACA  
 PDRCPTCIYVAGMAELGAFFSILQDMRNTIMASKTVGTAEELKKKSSFYQSYLRRTQSMGIQLDQRIILLYMLEWGKE  
 MVDHFHLGDDMDPELRGLAQLIDQKVKEISNQEPLKI

**The query sequence is most similar to:**  
*Puumala virus, strain P360 (Genbank accession L11347) with 100.0% similarity.*

**Similarity range:**  
 Mean similarity with group 1: 60.9% (59.5 – 62.0%)  
 Mean similarity with group 2: 90.9% (79.4 – 100.0%)  
 Mean similarity with group 3: 72.7% (70.2 – 73.7%)  
 Mean similarity with *Thottapalayam virus*: 46.4%

**Conclusion:**  
*The query sequence can be considered as a variant of Puumala virus strain A belonging to group 2*

**Files**

<b>Alignment file:</b>	getha7675.align
<b>Tree files:</b>	conclusion NJ tree ( <i>textfile</i> ) conclusion NJ tree ( <i>nexus format</i> ) conclusion NJ tree ( <i>PDF file</i> )

**FIGURE 3.1:** Screenshot of the result page. A Puumala virus sequence (Genbank accession number AB297665) was entered as query sequence. This query sequence was correctly identified by the GeTHa tool.

#### 4. RESULTS AND DISCUSSION

We have developed a web-based hantavirus classification tool that quickly and easily determines the correct genogroup and species of a query sequence. To validate the GeTHa tool, 500 nucleotide sequences of various lengths were selected from Genbank. After the determination of the correct species using the previous described guidelines for the genetic classification of hantaviruses (Chapter 2), these sequences were analyzed using the GeTHa

tool. For all hantavirus sequences with a length of 150 amino acids or more ( $n = 486$ ), the genogroup and species could be identified correctly. For the sequences with less than 150 amino acids ( $n = 14$ ) the genogroup could be distinguished correctly for most sequences (86%, 12/14), but only for 43% (6/14) of the sequences the correct species could be identified, which is in agreement with our previous findings (Chapter 2).

In comparison with the current classification of hantaviruses as defined by the International Committee on Taxonomy of Viruses which has 22 defined species (Nichol et al., 2005), our tool recognizes 15 of these species. The seven remaining species were reclassified as hantavirus strains in our genetic classification. Moreover, we identified three recently described hantavirus sequences (Sangassou virus, Tanganya virus and Vladivostok virus) as distinct hantavirus species (Chapter 2). Unfortunately, Tanganya virus has only one partial sequence available in Genbank with 147 amino acids in length (Klempa et al., 2007), which is not enough to function as a reference strain in the tool. Consequently, Tanganya virus sequences are currently not recognized as such.

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**DETECTION OF PUUMALA VIRUS ANTIBODY WITH ELISA  
USING A RECOMBINANT TRUNCATED NUCLEOCAPSID  
PROTEIN EXPRESSED IN *ESCHERICHIA COLI***

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## 1. SUMMARY

A truncated recombinant nucleocapsid protein (rNp118) consisting of the first 118 amino-terminal amino acids of the Puumala virus (PUUV) nucleocapsid protein expressed in *Escherichia coli* (*E. coli*), was evaluated for its antigenicity and reliability as serodiagnostic antigen in an enzyme-linked immunosorbent assay (ELISA) for detection of PUUV antibodies in human sera. The PUUV nucleocapsid protein has been shown to contain several B-cell epitopes, mapped within the first 118 amino-terminal amino acids. This finding makes the rNp118 an interesting recombinant protein to use as serodiagnostic antigen. The sensitivity of this new PUUV-rNp118 ELISA, was compared with those of a commercial available PUUV ELISA assay and an home-made ELISA based on a recombinant whole nucleocapsid protein of PUUV. Eighty-six human serum samples clinically suspected for PUUV-induced nephropathia epidemica (NE) and previously screened with the reference assays, were tested. The sensitivity of the new assay was compared with that of the reference assays and an excellent correlation between the assays was found. Sera found to be negative by other methods were also negative in our assay. The ELISA based on rNp118 represents an alternative and valid test for detection of antibodies to PUUV in human sera.

## 2. INTRODUCTION

PUUV carried by *Myodes glareolus* or bank vole, is the causative agent of nephropathia NE, a mild form of hemorrhagic fever with renal syndrome (HFRS) that occurs in western and central Europe and in western Russia (Brummer-Korvenkontio et al., 1980; Linderholm and Elgh, 2001; Maes et al., 2004). Clinical symptoms include fever, thrombocytopenia, several degrees of renal failure and, in severe cases, shock and haemorrhage. Several studies identified the PUUV nucleocapsid protein (Np) as a major antigenic target in early serological responses and demonstrated that high levels of PUUV-Np specific antibodies are produced during the acute phase of the disease (Niklasson and Kjellsson, 1988; Vapalahti et al., 1995; Lundkvist et al., 2002). The coding region of Np is relatively conserved and serological cross-reactivity between Np from other hantaviruses of the same genus is high (Elgh et al., 1998). The major antigenic domain for the humoral response to the Np protein is located at the amino-terminus (Elgh et al., 1996).

In this report, we cloned and expressed a truncated recombinant protein consisting of the first 118 amino-terminal aa (Puu118) in an *E. coli* mutant ICONE 200 (Chevalet et al., 2000) using

the tryptophan promoter controlled pTEXmp18 expression vector (Power et al., 1997). We tested rNp118 for its utility as serodiagnostic antigen in PUUV-infected patients in Belgium.

### 3. MATERIALS AND METHODS

#### 3.1 Construction of the expression plasmid

PUUV (strain CG1820) RNA was extracted directly from infected Vero E6 cells (CRL 1586, ATCC, USA) collected 7 days post-infection. The genomic RNA of PUUV was reverse-transcribed, and PCR amplified using One-step RT-PCR (Qiagen, Leusden, Belgium) in order to generate the entire S segment, using these oligonucleotide primers: 5'-CGG AAT TCA GTG ACT TGA CAG ACA TCC AAG-3' and 5'-CGA **AGC TTA** GTG GTG GTG GTG GTG GTG TAT CTT TAA GGG CTC CTG ATT TG-3'. The forward primer included an EcoRI site (underlined) and the reverse primer a HindIII site (bold and underlined) for subsequent cloning. The amino-terminal part encoding the first 118 aa was generated by PCR using 5'-GGT TCA CCG GTC TGT CGT CTA ACC GTG GTG GTG GTG GTG GTG **ATT CGA AGC**-3' as reverse primer. A protein tag (His) was introduced at the C-terminal end of the recombinant proteins, in order to provide a means of visualizing the specific protein by western blotting. RT-PCR and PCR fragments were cloned into the plasmid pTEXmp18 (Power et al., 1997). The generated constructions were sequenced using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Lennik, Belgium). DNA sequencing was performed with the Dye-terminator Rhodamine sequencing reaction mix (Applied Biosystems) and DNA sequences were analyzed using Sequencer 3.0 software (Applied Biosystems).

#### 3.2 Expression and purification of the recombinant protein

Following transformation of *E. coli* mutant ICONE 200, the recombinant proteins were produced as intracellular inclusion bodies, recovered, and renatured as previously described (Chevalet et al., 2000). Briefly the *E. coli* mutant ICONE 200 was grown in a 1 L Erlenmeyer flask containing 200 mL TSBY medium (30 g/L tryptic soy broth, 5 g/L yeast extract) and 8 µg/mL tetracycline at 37°C with vigorous shaking. Fermentations were carried out in a 3.5 L fermentor with 2 L medium. Cells were grown in batch conditions at 37°C and pH 7.0, while oxygen was maintained at 30% of saturation by automatic increase of agitation and then aeration through a cascade switch of the oxygen regulator on the speed and air-flow controllers. 3-β-indoleacrylic (IAA) was added at 5 to 25 µg/mL to induce expression. The

recombinant His-tagged proteins were extracted in denaturing conditions (6 M of guanidine-HCl). Purification by metal chelate affinity chromatography was achieved using the HisTrap kit (Pharmacia, Puurs, Belgium).

### **3.3 Western blotting**

The purified recombinant proteins were subjected to electrophoresis through 12% SDS-PAGE pre-cast Bis-Tris gel (Invitrogen, Leek, The Netherlands) and transferred to a PVDF (polyvinylidene difluoride) membrane (Invitrogen) using an electro-blotting apparatus (Bio-rad Laboratories, Nazareth Eke, Belgium). Immunoblotting was performed by using mouse anti-His (Sigma-Aldrich, Bornem, Belgium) or a PUUV-specific human serum sample (PCR positive, ELISA positive) and an ECL chemiluminescence detection kit (Amersham Biosciences, Roosendaal, The Netherlands) following the manufacturer's instructions.

### **3.4 Serum samples**

Eighty-six human serum samples of confirmed NE cases in Belgium, were screened in this study. The serum samples were already diagnosed to be either positive ( $n = 56$ ), borderline ( $n = 10$ ) or negative ( $n = 20$ ) for PUUV antibodies by a commercially available PUUV IgM/IgG ELISA assay (Progen, Heidelberg, Germany) and by a new in-house Puv-Np based ELISA carried out as described elsewhere except for the antigen preparation (Kallio-Kokko et al., 1998).

### **3.5 Enzyme-linked immunosorbent assay (ELISA)**

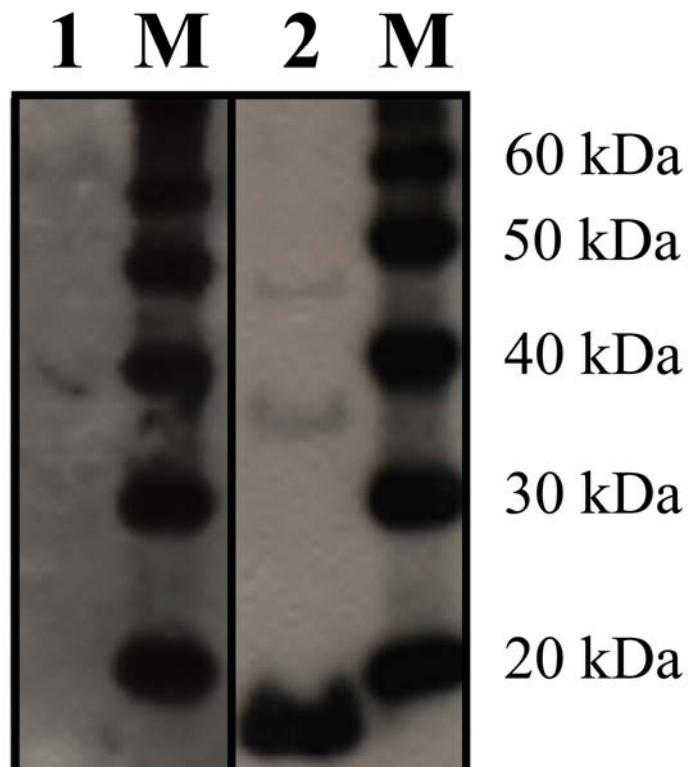
Maxisorp immuno-plates (Nalgene Europe, Neerijse, Belgium) were adsorbed overnight at 4°C with 5 µg of Puv118 or 10 µg of Puv-Np. Wells were subsequently blocked with a 2% solution of milk powder in PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) for 30 min. The plates were washed with PBS/0.05% Tween-20 and samples were added (1:400 dilution in PBS/0.05% Tween-20, pH 7.4) for 60 minutes. Washed plates were then incubated with 100 µL horseradish-peroxidase conjugated anti-human IgM or IgG antibodies (Southern Biotechnology Associates, Birmingham, USA). After 60 minutes, plates were washed and incubated with TMB membrane substrate (Calbiochem, San Diego, USA) for 10 minutes in the dark, and color development was stopped with an equal volume of 1N H<sub>2</sub>SO<sub>4</sub>. Absorbance was read at 450 nm against a reference wavelength of 620 nm, using a Multiskan MS ELISA plate reader (Thermo-Labsystems, Brussels, Belgium). The cut-off value was defined as the mean optical density (OD) plus three standard deviations, calculated from 20 PUUV negative

human sera samples from healthy volunteers used as negative controls. A sample was considered positive when showing an OD higher than the cut-off value.

## 4. RESULTS

### 4.1 Recombinant Protein

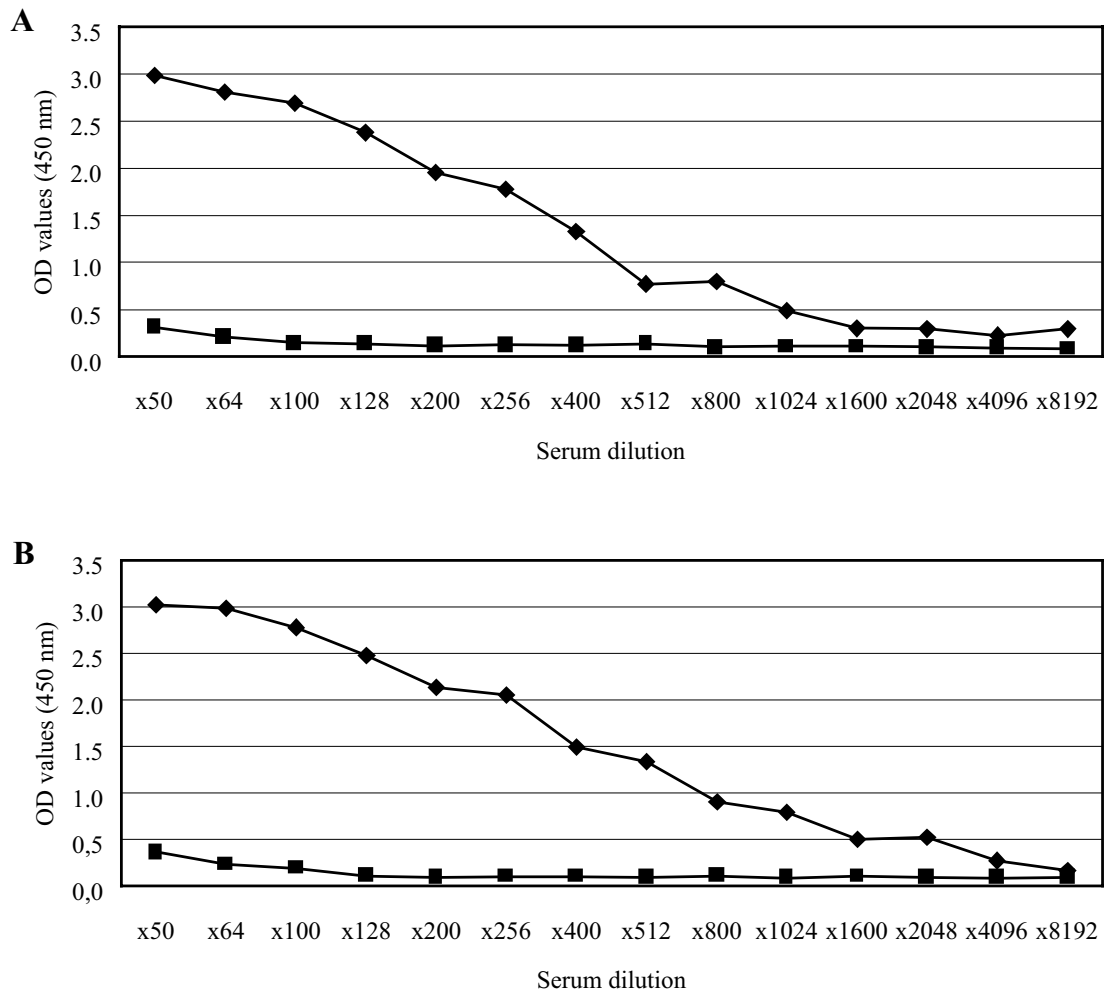
The sequence encoding the Np of PUUV was correctly cloned in the pTEX expression vector as confirmed by complete nucleotide sequencing, and the recombinant protein with a polyhistidine tag at the C-terminus was produced in *E. coli*. The recombinant-tagged protein was detected by western blotting using anti-His tag monoclonal antibody. In order to test the reactivity of Puu118, western blotting was carried out using both a PUUV positive (PCR positive and ELISA positive) and a PUUV negative human serum. The protein band of 18 kDa showed a strong and specific reaction with the PUUV positive serum whereas it was not recognized by the PUUV negative serum (FIGURE 4.1).



**FIGURE 4.1:** Western blotting analysis of the purified PUUV rNp-118 protein. Blotting was performed using a PUUV negative human serum (lane 1) and a PUUV positive human serum (lane 2). Lane 2 shows a protein band of 18 kDa, corresponding to the expected size of PUUV-rNp-118.

#### 4.2 *Puu118* ELISA

Initial assays were carried out to determine the optimal PUUV-rNp118 and PUUV-rNp concentrations and serum working dilution. The titration of the capture protein was optimized by checkerboard titration. An ELISA plate was coated with 5, 10, 15 or 20  $\mu\text{g}/\text{mL}$  protein concentration of Puu118 or Puu-Np and reacted with a PUUV-antibody positive human serum and PUUV-antibody negative human serum, serially diluted from 1:50 to 1:8192. An optimal concentration of 5  $\mu\text{g}/\text{mL}$  and 10  $\mu\text{g}/\text{mL}$  was considered for the capture proteins Puu118 and Puu-Np respectively. A dilution of 1:400 was adopted as serum dilution for Puu118 (FIGURE 4.2), and 1:200 for Puu-Np. A titration curve of IgM and IgG detection antibodies against dilutions of PUUV-antibody positive human serum was generated to determine the optimal level of detection and a titer of 1:5000 was adopted for both detection antibodies.



**FIGURE 4.2:** Response of 5  $\mu\text{g}/\text{ml}$  protein concentration of PUUV-rNp118 to PUUV positive human serum (◆) and PUUV negative human serum (■) detected with (A) anti-human IgG or (B) anti-human IgM.

To evaluate the precision of the ELISA, PUUV antibody positive human serum was assayed 10 times on one plate (within-run variation), and 10 times in 10 individual ELISA on different days (between-run and between-day variation). Plates were coated with the predetermined antigen concentration and serum dilutions. The average within-run, between-run and between-day variation were calculated according to the NCCLS EP5-A guideline (Hawker and Schlank, 2000) (TABLE 4.1).

To test the use of a Puu118 ELISA, 56 PUUV positive human sera (56 samples were positive for IgG, 42 samples were positive for IgM), 10 borderline positive sera and 20 negative human sera were tested. The cut-off value of ODs for the Puu118 IgG and IgM ELISA, were calculated as 0.302 (mean: 0.238, standard deviation: 0.021) and 0.408 (mean: 0.306, standard deviation: 0.034), respectively. For the Puu-Np IgG and IgM ELISA, the cut-off value was 0.521 (mean: 0.334, standard deviation: 0.063) and 0.517 (mean: 0.373, standard deviation: 0.048), respectively.

**TABLE 4.1** Within-run, between-run and between-day variation of the Puu118 ELISA.

	<b>IgM</b>	<b>IgG</b>
<b>Total variation (%)</b>	0.0660 (3.48)	0.0755 (3.71)
<b>Within-run variation (%)</b>	0.0559 (2.95)	0.0876 (4.31)
<b>Between-run variation (%)</b>	0.0376 (1.98)	0.0365 (1.36)
<b>Between-day variation (%)</b>	0.0324 (1.90)	0.0250 (1.23)

Fifty-six human sera which tested positive for IgG by the commercial hantavirus assay and by the Puu-Np ELISA (mean OD  $1.692 \pm 0.412$ ), were determined to be also positive by Puu118 ELISA (mean OD  $1.963 \pm 0.406$ ). Forty-two out of the fifty-six samples which tested positive for IgM by the reference tests also tested positive by Puu118 IgM ELISA (TABLE 4.2). For ten samples previously tested as weak positive (borderline) for IgM, 8 out of 10 tested positive with Puu118 IgM ELISA (mean OD  $1.670 \pm 0.135$ ), 2 tested weak positive (mean OD 0.929).

The results of the Puu118 ELISA correlate with the results of the reference tests, therefore the relative sensitivity and specificity remains unchanged.

**TABLE 4.2** Comparison of sensitivities of IgM PUUV-rNp118 ELISA, IgM PUUV-rNp ELISA and the commercial IgM Progen ELISA.

	PUUV-rNp118 ELISA (OD value)		PUUV-rNp ELISA (OD value)	
	pos.	neg.	pos.	neg.
Progen ELISA pos. (n=42)	42 (2.047 ± 0.208)	0	42 (1.757 ± 0.388)	0
Progen ELISA neg. (n=20)	0	20 (0.232 ± 0.019)	0	20 (0.347 ± 0.058)

## 5. DISCUSSION

PUUV is the causative agent of NE, a mild form of HFRS often associated with acute renal impairment. A rapid and reliable diagnosis of NE, commonly encountered in western and central Europe as well as in Russia, is important for differentiating NE from other infections having the kidney as target organ, this is particularly indicated in areas where PUUV is endemic, as in Belgium (Clement et al., 1997).

The use of Puu118 as antigen for ELISA, has the advantage in eliminating the risk in the processing of the antigen preparation. The preparation of life-virus antigen requires a biosafety level 3 containment laboratory due to the hazardous nature of the virus, while Puu118 is readily produced in a BSL-2 laboratory (Zoller et al., 1993; Yoshimatsu et al., 1993).

In this study Puu118 was evaluated for its antigenicity in an ELISA for PUUV IgM and IgG antibody detection. Previous studies have demonstrated the production of PUUV-Np specific antibodies during the acute phase of the disease, whereby detection of PUUV-Np specific IgM antibodies in clinical samples appeared to be good indicators of a recent PUUV infection (Vapalahti et al., 1995). Furthermore, the first 118 amino terminal aa of the Np, hold several B-cell epitopes, which makes this part one of the major targets of the immune system

(Lundkvist et al., 2002). Based on these findings, we cloned and expressed the Puu118 protein in *E. coli* for diagnostic use in ELISA.

The Puu118 protein was cloned and expressed in the *E. coli* ICONE 200 mutant using the pTEXmp18 expression vector which is controlled by a tryptophan promoter. Inducing expression using IPTG (isopropyl  $\beta$ -D-thiogalactopyranoside) in the BL21 *E. coli* strain for the expression of truncated PUUV rNp as proposed by Elgh and colleagues (Elgh et al., 1997), is an expensive and difficult procedure. Inducing expression with IAA using the *E. coli* mutant ICONE 200 is an alternative approach in expressing truncated PUUV Np with a reasonable cost.

The Puu118 protein was antigenically similar to the whole Np as shown by its specific reactivity with a human positive control serum in western blotting. The Puu118 ELISA was found to have a sensitivity similar to that of the commercial available hantavirus (Puumala) IgM/IgG ELISA and the Puu-Np ELISA since all gave concordant results. Weakly positive samples gave relatively higher OD values with our newly developed ELISA than with both reference assays. The reference assays both had a capture protein concentration of 10  $\mu$ g and a serum dilution of 1:200, whereas for the Puu118 ELISA these were 5  $\mu$ g and 1:400 respectively, demonstrating that less antigen is needed for the Puu118 ELISA than for the reference tests.

Truncated Np were previously proven highly sensitive for diagnostic use in an ELISA format, and in most cases specific enough to differentiate the involved hantavirus serotype, i.e. to allow a distinction (higher homologous OD) between infections caused by PUUV, Hantaan virus, Seoul virus, or Dobrava virus as proven by neutralization testing (Araki et al., 2001), or by PCR testing (Miyamoto et al., 2003). Japanese workers described already in 1995 higher sensitivity and specificity with Np than with classic IFA for serodiagnosis of Hantaan virus infection in experimentally infected rats (Yoshimatsu et al., 1995). *E. coli* expressed Np of five different pathogenic hantavirus strains used in ELISA were subsequently shown to have indeed a high degree of correlation with results obtained with other diagnostic methods (Elgh et al., 1997). These data, together with our current findings indicate that the Puu118 protein is a useful antigen for reliable and rapid detection of specific PUUV antibodies with ELISA.

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**REPLICATION REDUCTION NEUTRALIZATION TEST, A  
QUANTITATIVE RT-PCR-BASED TECHNIQUE FOR THE  
DETECTION OF NEUTRALIZING HANTAVIRUS ANTIBODIES**

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This chapter is submitted to:

**Piet Maes, Els Keyaerts, Sandra Li, Véronique Nlandu-Masunda, Jan Clement, and Marc Van Ranst.** 2007. Replication Reduction Neutralization Test, a quantitative RT-PCR-based technique for the detection of neutralizing hantavirus antibodies. *Journal of Virological Methods*, submitted.

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## 1. SUMMARY

A hantavirus neutralization test based on quantitative RT-PCR (qRT-PCR) was developed to detect the degree of reduction in virus replication after incubation with sera containing specific neutralizing antibodies. Using the qRT-PCR, hantavirus genomes as low as 100 genomes/reaction could be detected, making qRT-PCR a very sensitive tool for hantavirus neutralization tests. The replication reduction neutralization test (RRNT) can be performed using 96-well culture plates, minimizing the use of cells and reagents. Moreover, the detection of hantavirus genomes with qRT-PCR eliminates the need for animal anti-hantavirus antibodies to detect the residual hantavirus genomes as needed for focus reduction neutralization test (FRNT). To evaluate the effectiveness of RRNT, several hantaviruses were used to test hantavirus-specific convalescent human or mouse serum samples. These serum samples were shown to contain neutralizing hantavirus antibodies by FRNT. All convalescent serum samples tested by RRNT showed significant decreases in hantavirus genomes for only one specific hantavirus species, thus allowing an easy identification of the infectious virus. The results obtained by RRNT were 100% comparable with the results acquired by FRNT. In conclusion, an *in vitro* RRNT approach as a reliable and quick alternative for FRNT, hitherto considered as the gold standard in hantavirus serology.

## 2. INTRODUCTION

The rodent-borne hantaviruses belonging to the family Bunyaviridae, are a group of enveloped negative-sense RNA viruses, causing the human diseases known as hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia, and hantavirus cardiopulmonary syndrome (HCPS) in the Americas (Lahdevirta, 1971; Schmaljohn and Hjelle, 1997; Clement et al., 1997; Kruger et al., 2001). The genome organization of hantaviruses is typical of other members of the family Bunyaviridae, consisting of three negative-stranded RNA segments with a large L segment encoding the viral RNA polymerase, a medium M segment encoding the envelope glycoproteins G1 and G2, and a small S segment encoding the viral nucleocapsid protein (Schmaljohn and Dalrymple, 1983; Plyusnin et al., 1996; Maes et al., 2004). A common feature of hantavirus diseases is the increased permeability of the vascular endothelium, suggesting that the vascular endothelium is a prime target for virus infection (Markotic et al., 2003). Endothelial cells are susceptible to hantavirus infection, however, hantaviruses do not generally cause cytopathic effects to explain the increase in endothelial

permeability (Markotic et al., 2003). Although the lack of a visible cytopathic effect, makes it difficult to perform plaque-reduction neutralization tests with hantaviruses, this test is the most specific test to detect neutralizing antibodies elicited by infection or vaccination and for discriminating different strains of hantaviruses. However, not all hantaviruses form plaques. By using the focus reduction neutralization test, the hantavirus plaques are visualized with immunochemical staining (Tanishita et al., 1984; Niklasson et al., 1991). The foci can be observed at low magnification under a dissecting microscope, what makes counting of the foci easy and accurate. Moreover, the detection of the foci by chemiluminescence makes the focus reduction neutralization test even more sensitive than by using conventional immunostaining (Heider et al., 2001).

Although neutralizing antibodies develop early after infection and are habitually already present at the onset of disease (Horling et al., 1992), acute and early convalescent phase sera can usually not be used for identification of the causative hantavirus (Lundkvist et al., 1997). Cross-reactions in the early phase of the disease are probably due to the presence of high cross-neutralizing titers of IgM antibodies. Neutralizing antibodies to the M segment (G1 and G2 glycoproteins), but not to the S segment (nucleocapsid protein), have been identified. Until now, nothing is known about the serological responses to the L segment of hantaviruses. The purpose of this study was to develop a sensitive, quantitative assay for hantavirus neutralizing capacities of immune sera. We developed an *in vitro* hantavirus neutralization test, the replication reduction neutralization test, using a quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)-based system.

### **3. MATERIALS AND METHODS**

#### **3.1 *Virus and cell culture***

The virus strains used in this study were Andes virus (ANDV) strain Chile-9717869 (Toro et al., 1998), Dobrava-Belgrade virus (DOBV) strain 90/5 (Avsic-Zupanc et al., 1992), DOBV strain SK/Aa (Klempa et al., 2005), Hantaan virus (HTNV) strain 76-118 (Lee et al., 1978), Puumala virus (PUUV) strain CG1820 (Stohwasser et al., 1990), Seoul virus (SEOV) strain R22 (Shi et al., 1998), SEOV strain Tchoupitoulas 579-8 (Tsai et al., 1985), Sin Nombre virus (SNV) strain NMR11 (Chizhikov et al., 1995), Thottapalayam virus (TMPV) (Carey et al., 1971), and Tula virus (TULV) strain Malacky (Sibold et al., 1995). Vero E6 cells (American Type Culture Collection, C1008) were cultured in minimum essential medium (MEM)

supplemented with 10% heat-inactivated fetal calf serum. The maintenance medium for virus propagation was identical but contained 2% fetal calf serum. All hantaviruses used in this study were propagated for 10 days on monolayers of Vero E6 cells, except DOBV 90/5 which was propagated for 16 days. Cells and viruses were cultured at 37°C with 5% CO<sub>2</sub>.

### **3.2 Serum samples**

Human hantavirus IgG positive, convalescent serum samples of confirmed hantavirus cases were screened in this study (5 DOBV, HTNV, PUUV and SEOV serum samples). For each hantavirus strain used for this study, 3 mouse serum samples containing neutralizing antibodies against the specific hantavirus strain, were screened. These serum samples were obtained by immunizing 6-week-old C57Bl/6 mice (Elevage Janvier, Le Genest Saint Isle, France) four times intraperitoneally (3 weeks interval) with inactivated and purified hantavirus dilutions. Blood was drawn 3 weeks after the last immunization and serum was collected. Five serum samples obtained from five untreated C57Bl/6 mice were used as negative controls in the tests. Serum samples were heat-inactivated for 30 minutes at 56°C before use. We adhered to ethical standards for manipulating the animals. An approval from the Ethics Committee of the K.U. Leuven was obtained.

### **3.3 Primer and fluorogenic probe design**

The S segment nucleocapsid gene of the different hantavirus strains was searched for primer and probe target sites that would be compatible with Taqman PCR requirements (ABI 7500 Fast Users Manual) by using Primer Express version 2.0 software. The primers had matched dissociation temperatures and a minimal likelihood for duplex or hairpin formation. Primer and probe sequences are summarized in TABLE 5.1. The Taqman probes were FAM-TAMRA labeled. The Tula virus forward and reverse primers are derived from the M segment.

### **3.4 Hantavirus quantitation**

Specific cRNA standards for each virus were used to calculate the exact viral copy numbers. The cRNA standards were made using the MEGAshortscript<sup>TM</sup> T7 High Yield Transcription kit (Ambion, Austin, Texas, USA) as described previously (Maes et al., 2006). Briefly, PCR products amplified with a modified forward primer (addition of T7-promotor TAA TAC GAC TCA CTA TAG GGA GG at the 5' end of the initial forward primer) and corresponding reverse primer were quantified spectrophotometrically at 260 nm. After *in vitro* transcription and purification, quantitation of the cRNA-standards was done spectrophotometrically at 260 nm. The

measurements of cRNA concentration were undertaken in duplicate and then converted to the molecule number (Fronhoffs et al., 2002).

**TABLE 5.1:** Primer and Taqman probe sequences.

	<b>Forward primer (5' to 3')</b>	<b>Reverse primer (5' to 3')</b>	<b>Taqman probe (5' to 3')</b>
<b>ANDV</b>	CCCATTTCAGTCTGGGTGTTTG	GTACCCCTGCAACATACAATGC	TGTGCACCTGACCGGTGCCC
<b>DOBV</b>	TCCCGTGCAAGCTACTATCTGA	GCGCTCCTTGTCTTTGATTCA	ACCAAAGGCCCATCCACCAATCGT
<b>HTNV</b>	CTGGATTTAAACCATTTGGATATTGA	TATCGGGACGACAAAAGGATGTA	AGCAGACTGGCTGAGCATCATCGTCTATCT
<b>PUUV</b>	TACAAGAGAAGAATGGCAGATGCT	CATTACATCAAGGACATTTCCA	CTGACCCGACTGGGATTGAACCTGA
<b>SEOV</b>	TGTCAATGGAATCAGAAAAGCCCAA	CCAGGTGTTATCTCTTCAGCCTTC	TGTGTCAATGCCAAACGCCCAATCC
<b>SNV</b>	TACTCCTCATTTCAGTTTGGGTCTTT	TCCCGGCCACATA TAATGCT	CATGTGCTCCAGATCGTTGTCCACCT
<b>TMPV</b>	AGAAGCCACCCCATGGTTATT	GGATACCTGCCACTTGAAGAACA	ATGCACCAGACCGATGCCCTCCT
<b>TULV</b>	CCAGGTGTTGCACATTCTCTTG	GAGGAATAGCTAGCCAGCCAAA	TGAATTATGTGTTTCCTGGGCTGCATGG

### 3.5 Quantitative RT-PCR (qRT-PCR)

qRT-PCR was carried out using the Eurogentec One Step RT qPCR kit (Eurogentec, Seraing, Belgium) with the ABI Prism 7500 Fast Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as described previously (Maes et al., 2006). Briefly, the reaction was conducted in a 25 µl volume containing 5 µl of extracted RNA, 12.5 µl of One step RT qPCR MasterMix (Eurogentec, Seraing, Belgium) containing ROX as a passive reference, 900 nM forward and reverse primer, 250 nM probe, which was labeled at the 5' end with the fluorescent dye 6-carboxyfluorescein (FAM) as the reporter dye, and the 3' end was labeled with quencher dye 6-carboxytetramethylrhodamine (TAMRA), and 0.125 µl Euroscript/RNase inhibitor (Eurogentec). Reverse transcription was initiated at 48°C for 30 min, followed by PCR activation at 95°C for 10 min and 45 cycles of a two-step incubation at 95°C for 15 s and 60°C for 1 min. The reporter dye (FAM) signal was measured against the internal reference dye (ROX) to normalize for non-PCR-related fluorescence emissions. The threshold cycle ( $C_T$ ) was defined as the fractional cycle number at which the reporter fluorescence, generated by cleavage of the probe, reaches a threshold defined as 10 times the standard deviation of the mean baseline emission.

### **3.6 Replication reduction neutralization test (RRNT)**

The replication reduction neutralization test was carried out in 96-well plates. All dilutions of sera and viruses were carried out by using minimum essential medium (MEM) supplemented with 2% heat-inactivated fetal calf serum. Virus ( $2 \times 10^3$  hantavirus copies/ml) was mixed with an equal volume of a serum dilution (from 1:20 to 1:25,600). After a pre-incubation of 1h at 37°C in a humidified 5% CO<sub>2</sub> atmosphere, 20 µl/well of the virus/antibody mixtures were added to a 96-well plate containing confluent Vero E6 cell monolayers and incubated for 1h at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. For each serum sample tested, four wells were infected with virus incubated with medium alone (non-neutralized virus) as controls and every serum dilution was run in twofold. After the incubation period, 180 µl of pre-warmed (37°C) MEM was added to each well and plates were again incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. After an incubation of 10 days, viral RNA was extracted by using the QIAamp viral RNA kit (Qiagen, Leusden, The Netherlands). The titers of virus were determined by qRT-PCR. An 80% reduction in the number of hantavirus copies in comparison with a non-neutralized virus control (cells infected with hantavirus, incubated with MEM supplemented with 2% heat-inactivated fetal calf serum) was used as the criterion for virus neutralization titers.

### **3.7 Focus reduction neutralization test (FRNT)**

The focus reduction neutralization test was carried out as described elsewhere (Tanishita et al., 1984; Niklasson et al., 1991). Briefly, sera were serially diluted and mixed with an equal volume containing 30 to 50 focus forming units (FFU) of virus per 100 µl. The mixtures were incubated for 60 minutes and inoculated subsequently into 6-well plates containing confluent Vero E6 cell monolayers. After adsorption for 1h, the wells were overlaid with a mixture of agarose (1%) and basal Eagle's medium. Plates were incubated for 6 days (HTNV), 7 days (SEOV and TULV), or 10 days (DOBV, PUUV, SNV). Virus-infected cells were detected with hantavirus-specific rabbit polyclonal antisera (kindly provided by Dr. D.H. Krüger, Institut für Virologie, Charité, Berlin, Germany), followed by peroxidase-labeled goat anti-rabbit antibodies and substrate.

### **3.8 Statistical analysis**

To determine whether the number of hantavirus genomes from cells infected with virus incubated with serum was significantly lower than the number of hantavirus genomes from the controls (hantavirus-infected cells incubated with MEM supplemented with 2% heat-

inactivated fetal calf serum), the Student's *t*-test was calculated based on unequal variances with a one-tailed critical *P* value of 0.05.

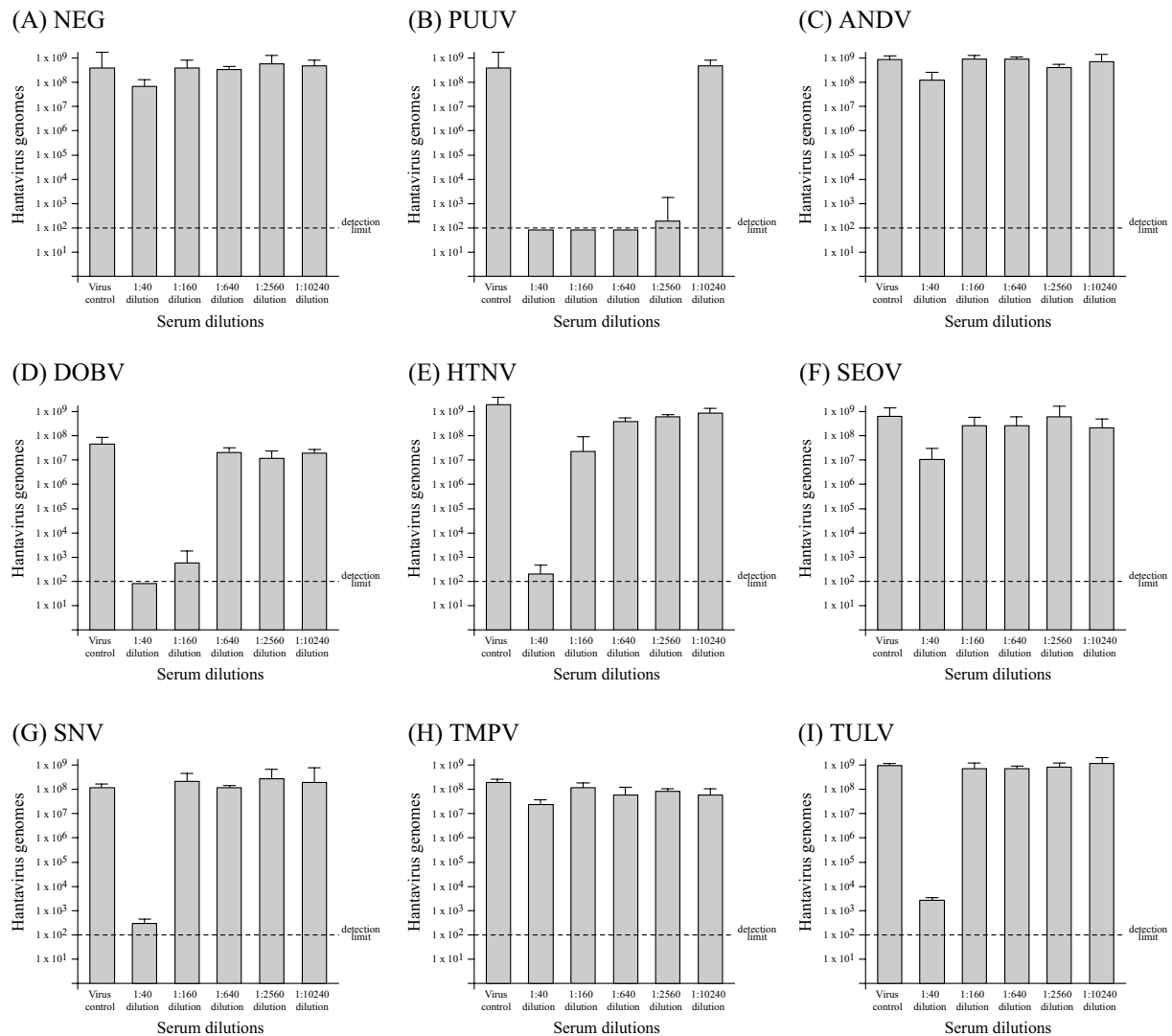
## 4. RESULTS

### 4.1 *Standardization and quantitation of qRT-PCR*

To determine the absolute quantity of virus copies in a sample, hantavirus cRNA standards were used for the generation of standard curves. Serial dilutions of the standards were made and corresponding copy numbers of specific transcripts from  $10^2$  up to  $10^9$  were used. Equal volumes of standard and sample were used for PCR amplification. The Sequence Detector v1.9 software (Applied Biosystems) was used for the analysis of the copy numbers and linear regression curve. The dynamic ranges of the different hantavirus assays were determined by testing 10-fold serial dilutions of the cRNA standard ranging from 10 to  $10^{12}$  molecules. The results were analyzed in terms of  $C_T$  value (the cycle in which a target sequence was detected). The dynamic range of the assays spans 6 logs ranging from 2 to 8 log hantavirus copies per reaction, corresponding to main  $C_T$  values ranging from  $38.74 \pm 0.47$  to  $14.32 \pm 0.31$ .

### 4.2 *Optimization and application of qRT-PCR to virus neutralization test*

We supposed qRT-PCR could be used to demonstrate the reduction in hantavirus replication resulting from neutralization by virus-neutralizing antibodies present or not in the tested sera. To test this, we performed virus neutralization tests by using specific immune mouse sera for every hantavirus strain and evaluated the results both by qRT-PCR (replication reduction neutralization test, RRNT) and focus reduction neutralization test (FRNT) (TABLE 5.2 and FIGURE 5.1). The neutralization was done with serial serum dilutions ranging from 1:20 to 1:25,600. For both RRNT and FRNT, the highest serum dilution which still showed specific virus neutralization was 1:2,560. The lowest dilution tested, 1:20, showed high cross-reaction between sera originating from different hantavirus species (heterologous reactions). This cross-reaction declined quickly with higher serum dilutions. For all mouse sera tested in this study, no significant reduction in virus growth could be detected with heterologous reaction of 1:160 and 1:320 dilutions for respectively RRNT and FRNT. The standard serum dilutions for the RRNT were set to 1:40, 1:160, 1:640, 1:2,560, and 1:10,240.

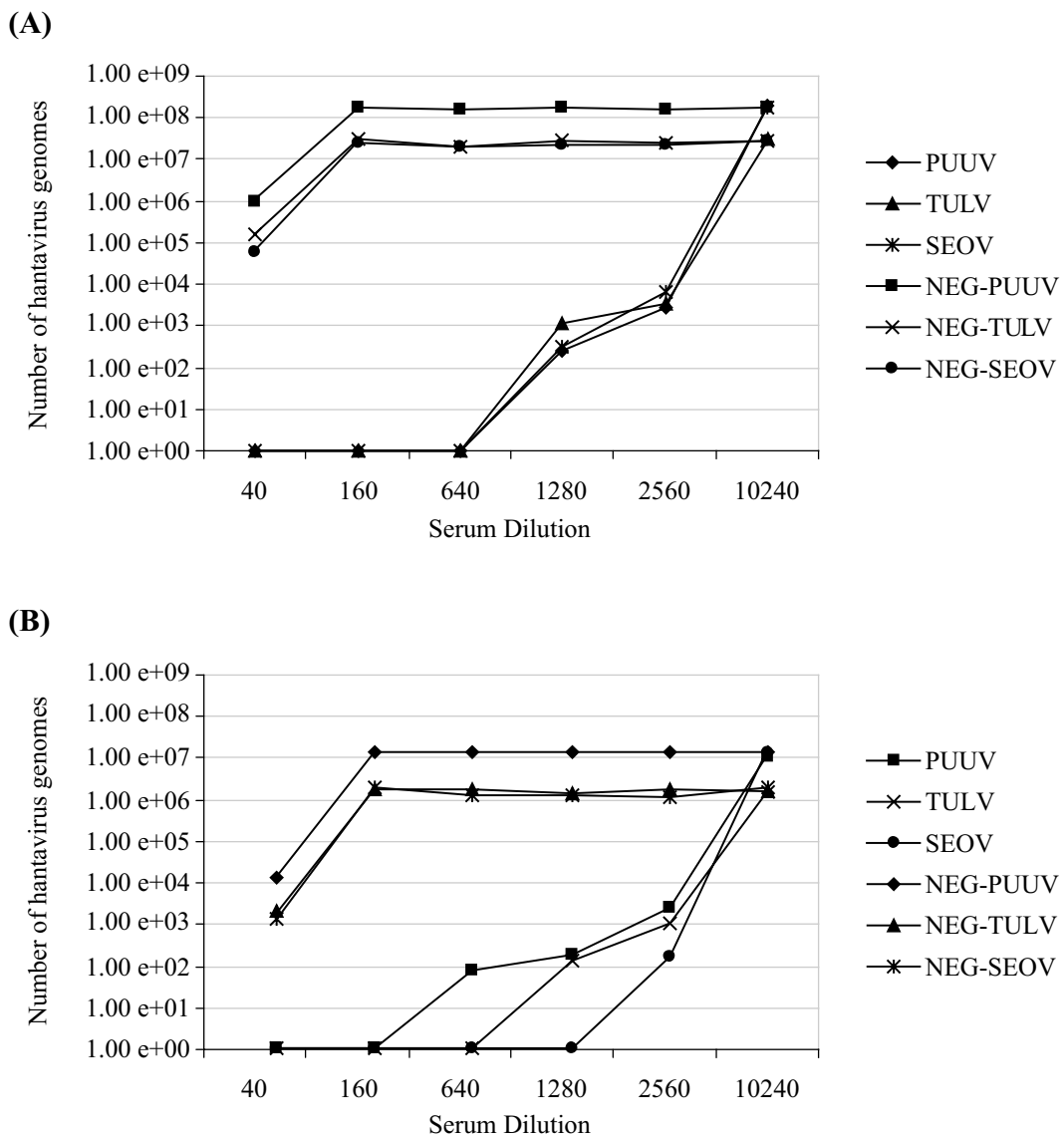


**FIGURE 5.1:** Absolute hantavirus genomes versus serum dilution of a replication reduction neutralization test performed with a Puumala virus specific mouse serum sample. Virus control consists of virus incubated with MEM 2%. (A) Puumala virus incubated with a hantavirus negative serum, (B) Puumala virus, (C) Andes virus, (D) Dobrava-Belgrade virus, (E) Hantaan virus, (F) Seoul virus, (G) Sin Nombre virus, (H) Thottapalayam virus, (I) Tula virus.

The optimal virus starting concentration for FRNT is 30 to 50 foci per well (Heider et al., 2001). To assess the optimal virus concentration for RRNT, multiple virus concentrations ranging from 10 to  $5 \times 10^4$  hantavirus copies per well were tested. For all hantavirus strains except DOBV strain 90/5, the optimal concentration was found to be 50 hantavirus genome copies per well (FIGURE 5.2). For DOBV strain 90/5 the optimal starting concentration was found to be 150 hantavirus genome copies per well.

All hantavirus strains except DOBV strain 90/5, had to be incubated for 10 days to have sufficient high virus titers in the controls (cells infected with 50 hantavirus copies per well, incubated with MEM supplemented with 2% heat-inactivated fetal calf serum). DOBV strain

90/5 had to grow for 12 days to have sufficient virus titers. Furthermore, comparison of the initial numbers of hantavirus genome copies added to each well with the numbers of genome copies obtained after a 10 days incubation (or 12 days incubation for DOBV strain 90/5) in the absence of neutralizing serum, showed an at least 10,000-fold increase. This, together with the low starting concentrations of the viruses, which are below the detection limit of the qRT-PCR, indicates that genomes of neutralized hantaviruses in the medium do not interfere with detection of reduction of viral replication by neutralizing antibody.



**FIGURE 5.2:** Virus neutralization evaluated by qRT-PCR by using Puumala virus (PUUV), Seoul virus (SEOV) or Tula virus (TULV). Hantavirus-positive sera (PUUV, SEOV and TULV) and hantavirus-negative sera (NEG-PUUV, NEG-SEOV and NEG-TULV) were tested in virus neutralization assays in different serum dilutions by using (A) 500 or (B) 50 hantavirus genomes per well. The reduction of viral genomes by the negative sera at the 1:40 dilution was not statistically significant (one-tailed t-test).

**4.3 Neutralization test using qRT-PCR with mouse sera and comparison to FRNT**

For each of the ten hantavirus strains used in this study, three C57Bl/6 mice were immunized four times with purified and inactivated virus to make specific mouse immune serum. These serum samples were evaluated with RRNT and FRNT using each hantavirus strain. Homologous neutralizing titers were at least two fold higher than heterologous neutralizing titers for both RRNT and FRNT (TABLE 5.2). The neutralizing titers, based on an 80% reduction of infectivity were equal for FRNT and RRNT.

When analyzing different strains of the same hantavirus species, which was done for DOBV and SEOV, strains 90/5 and SK/Aa, and strains Tchoupitoulas 579-8 and R22 for respectively DOBV and SEOV, no distinction could be made in neutralizing titers between the different strains for both RRNT and FRNT (TABLE 5.2).

To confirm that an 80% reduction of infectivity indicates the neutralizing titers, five negative serum samples derived from 6-weeks-old healthy C57Bl/6 mice were additionally analyzed using RRNT. These sera were hantavirus antibody negative when tested by IgM/IgG ELISA (Progen, Heidelberg, Germany) with goat anti-mouse IgM/IgG specific detection antigens (Southern Biotechnology Associates, Birmingham, USA) and all showed neutralizing titers equal too or below 1:40 (TABLE 5.2).

**4.4 Neutralization test using qRT-PCR with HFRS patient sera and comparison to FRNT**

To examine the applicability of the neutralization test for measuring the neutralizing value of human sera, we compared the neutralizing titers of the RRNT and FRNT by using representative patient serum samples of each HFRS serotype (DOBV, HTNV, PUUV and SEOV). These sera neutralized the homologous hantaviruses in a dilution factor-dependent manner. The neutralization titers obtained with RRNT and FRNT were comparable with titers of the homologous reactions roughly two to three times higher than the titers of the heterologous reactions (TABLE 5.3).

**TABLE 5.2:** RRNT and FRNT neutralization titers of sera from mice evaluated with different hantaviruses. The highest neutralizing reciprocal endpoint titer found is indicated in bold for each sample. NEG: hantavirus negative serum, DOBV1: Dobrava-Belgrade virus strain 90/5, DOBV2: Dobrava Belgrade virus strain SK/Aa, SEOV1: Seoul virus strain R22, SEOV2: Seoul virus strain Tchoupitoulas 579-8.

Serum number		RRNT neutralization titers ( <i>FRNT neutralization titers</i> )									
		ANDV	DOBV1	DOBV2	HTNV	PUUV	SEOV1	SEOV2	SNV	TMPV	TULV
NEG	1	40 (40)	<40 (40)	<40 (40)	<40 (40)	<40 (40)	40 (40)	<40 (<40)	<40 (<40)	40 (<40)	<40 (<40)
	2	40 (<40)	40 (40)	40 (40)	<40 (<40)	<40 (<40)	<40 (<40)	160 (<40)	40 (<40)	<40 (<40)	40 (<40)
	3	<40 (<40)	<40 (<40)	<40 (40)	<40 (<40)	<40 (<40)	<40 (<40)	<40 (<40)	<40 (<40)	<40 (<40)	<40 (40)
ANDV	1	<b>2560</b> (640)	<40 (160)	<40 (40)	<40 (160)	<40 (<40)	<40 (<40)	<40 (<40)	<40 (160)	<40 (40)	<40 (<40)
	2	<b>640</b> (640)	<40 (40)	<40 (40)	<40 (40)	<40 (<40)	<40 (40)	<40 (160)	<40 (<40)	<40 (40)	<40 (40)
	3	<b>640</b> (640)	<40 (40)	<40 (40)	<40 (<40)	<40 (<40)	<40 (40)	<40 (<40)	<40 (<40)	<40 (<40)	<40 (40)
DOBV1	1	<40 (<40)	<b>640</b> (640)	<b>640</b> (2560)	160 (40)	40 (<40)	40 (<40)	<40 (40)	<40 (<40)	<40 (<40)	<40 (40)
	2	40 (<40)	<b>640</b> (640)	<b>640</b> (2560)	40 (<40)	40 (<40)	40 (<40)	160 (40)	<40 (40)	<40 (40)	40 (<40)
	3	<40 (<40)	<b>2560</b> (10240)	<b>2560</b> (640)	160 (40)	<40 (<40)	<40 (40)	<40 (<40)	<40 (<40)	<40 (160)	<40 (<40)
DOBV2	1	<40 (40)	<b>640</b> (2560)	<b>2560</b> (2560)	<40 (<40)	160 (<40)	<40 (160)	40 (<40)	160 (160)	<40 (<40)	<40 (<40)
	2	<40 (160)	<b>640</b> (640)	<b>640</b> (640)	<40 (<40)	<40 (<40)	40 (<40)	40 (160)	40 (40)	<40 (<40)	<40 (<40)
	3	<40 (40)	<b>640</b> (640)	<b>640</b> (640)	<40 (<40)	160 (40)	160 (40)	<40 (<40)	40 (<40)	<40 (<40)	<40 (<40)
HTNV	1	<40 (40)	<40 (<40)	<40 (40)	<b>2560</b> (640)	40 (40)	<40 (<40)	40 (<40)	40 (160)	<40 (<40)	160 (40)
	2	<40 (40)	40 (<40)	<40 (40)	<b>2560</b> (2560)	40 (<40)	<40 (<40)	40 (40)	40 (<40)	<40 (40)	<40 (40)
	3	<40 (<40)	<40 (<40)	<40 (<40)	<b>10240</b> (2560)	160 (40)	<40 (40)	<40 (<40)	160 (40)	<40 (<40)	<40 (<40)
PUUV	1	<40 (40)	160 (40)	<40 (<40)	160 (160)	<b>2560</b> (2560)	<40 (40)	<40 (<40)	<40 (<40)	<40 (<40)	160 (40)
	2	<40 (<40)	<40 (<40)	<40 (<40)	160 (160)	<b>10240</b> (2560)	<40 (<40)	40 (40)	<40 (<40)	<40 (40)	40 (40)
	3	<40 (<40)	40 (<40)	40 (<40)	40 (160)	<b>10240</b> (2560)	<40 (40)	40 (<40)	40 (<40)	<40 (40)	40 (<40)
SEOV	1	<40 (<40)	<40 (40)	160 (40)	40 (<40)	<40 (40)	<b>2560</b> (640)	<b>2560</b> (640)	<40 (<40)	40 (40)	40 (160)
	2	<40 (<40)	<40 (<40)	<40 (<40)	40 (<40)	<40 (<40)	<b>2560</b> (2560)	<b>2560</b> (2560)	<40 (<40)	<40 (<40)	<40 (40)
	3	<40 (160)	<40 (<40)	<40 (<40)	<40 (<40)	<40 (40)	<b>10240</b> (2560)	<b>10240</b> (2560)	<40 (<40)	40 (<40)	<40 (<40)
SEOV	1	<40 (40)	40 (<40)	40 (<40)	40 (<40)	<40 (40)	<b>2560</b> (2560)	<b>2560</b> (2560)	<40 (40)	<40 (<40)	<40 (<40)
	2	<40	<40	<40	40	40	<b>640</b>	<b>640</b>	<40	<40	40

	3	( <i>&lt;40</i> ) <40 ( <i>&lt;40</i> )	( <i>&lt;40</i> ) <40 ( <i>&lt;40</i> )	( <i>&lt;40</i> ) <40 ( <i>&lt;40</i> )	( <i>40</i> ) 160 ( <i>40</i> )	( <i>40</i> ) 40 ( <i>&lt;40</i> )	<b>(640)</b> <b>2560</b> <b>(2560)</b>	<b>(640)</b> <b>2560</b> <b>(2560)</b>	( <i>&lt;40</i> ) <40 ( <i>&lt;40</i> )	( <i>&lt;40</i> ) <40 ( <i>&lt;40</i> )	( <i>&lt;40</i> ) <40 ( <i>&lt;40</i> )
<b>SNV</b>	1	<40 ( <i>40</i> )	40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	40 ( <i>&lt;40</i> )	40 ( <i>160</i> )	40 ( <i>40</i> )	<40 ( <i>&lt;40</i> )	<b>640</b> <b>(2560)</b>	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )
	2	<40 ( <i>&lt;40</i> )	160 ( <i>40</i> )	<40 ( <i>&lt;40</i> )	40 ( <i>&lt;40</i> )	40 ( <i>40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<b>640</b> <b>(640)</b>	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )
	3	<40 ( <i>40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>40</i> )	40 ( <i>&lt;40</i> )	<b>640</b> <b>(640)</b>	<40 ( <i>&lt;40</i> )	40 ( <i>160</i> )
	1	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	40 ( <i>&lt;40</i> )	<b>640</b> <b>(2560)</b>	<40 ( <i>&lt;40</i> )
	2	<40 ( <i>40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>40</i> )	<40 ( <i>&lt;40</i> )	40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<b>2560</b> <b>(2560)</b>	<40 ( <i>&lt;40</i> )
	3	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>40</i> )	40 ( <i>&lt;40</i> )	<b>640</b> <b>(640)</b>	<40 ( <i>40</i> )
<b>TULV</b>	1	<40 ( <i>40</i> )	<40 ( <i>40</i> )	<40 ( <i>40</i> )	<40 ( <i>&lt;40</i> )	40 ( <i>160</i> )	<40 ( <i>40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	40 ( <i>&lt;40</i> )	<b>2560</b> <b>(640)</b>
	2	<40 ( <i>&lt;40</i> )	<40 ( <i>40</i> )	<40 ( <i>&lt;40</i> )	40 ( <i>160</i> )	40 ( <i>&lt;40</i> )	40 ( <i>&lt;40</i> )	40 ( <i>160</i> )	40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<b>2560</b> <b>(2560)</b>
	3	<40 ( <i>&lt;40</i> )	160 ( <i>40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	160 ( <i>160</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<b>10240</b> <b>(2560)</b>
	1	<40 ( <i>40</i> )	<40 ( <i>40</i> )	<40 ( <i>40</i> )	<40 ( <i>&lt;40</i> )	40 ( <i>160</i> )	<40 ( <i>40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	40 ( <i>&lt;40</i> )	<b>2560</b> <b>(640)</b>
	2	<40 ( <i>&lt;40</i> )	<40 ( <i>40</i> )	<40 ( <i>&lt;40</i> )	40 ( <i>160</i> )	40 ( <i>&lt;40</i> )	40 ( <i>&lt;40</i> )	40 ( <i>160</i> )	40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<b>2560</b> <b>(2560)</b>
	3	<40 ( <i>&lt;40</i> )	160 ( <i>40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	160 ( <i>160</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<40 ( <i>&lt;40</i> )	<b>10240</b> <b>(2560)</b>

## 5. DISCUSSION

A hantavirus neutralization test based on qRT-PCR to detect reduction in virus replicated after neutralization, was developed to detect and titer anti-hantavirus-neutralizing antibodies. By using the qRT-PCR assay, hantavirus genomes as low as  $10^3$  genomes could be detected, making qRT-PCR a sensitive tool for hantavirus neutralization tests. The replication reduction neutralization test (RRNT) can be performed in 96-well culture plates, which minimized the use of cells and reagents. Moreover, the detection of hantavirus genomes with qRT-PCR eliminates the need for animal anti-hantavirus antibodies to detect the residual hantavirus genomes as needed for FRNT. Furthermore, the results obtained with qRT-PCR are analyzed in an automated fashion, in contrast to FRNT where foci are counted using a dissecting microscope, which is labor intensive. A recognized problem with qRT-PCR however, is that the presence of viral RNA does not necessarily point to the presence of infectious virus (Gassilloud et al., 2003; Suarez et al., 2003). By using starting concentrations for the RRNT assay below the detection limit of the qRT-PCR (50 hantavirus genomes per reaction), hantavirus genomes detected after the neutralization assay all come from infectious viruses that were not neutralized by the sera tested. Using this approach, interference of residual RNA originating from inactivated virus can be diminished.

**TABLE 5.3:** RRNT and FRNT neutralization titers of sera from HFRS patients. The highest neutralizing reciprocal endpoint titer found is indicated in bold for each sample. DOBV: Dobrava-Belgrade virus strain 90/5, HTNV: Hantaan virus strain 78/118, PUUV: Puumala virus strain CG1822, SEOV: Seoul virus R22.

Serum number	RRNT neutralization titers ( <i>FRNT neutralization titers</i> )				
	DOBV	HTNV	PUUV	SEOV	
<b>DOBV</b> 1	<b>640</b>	160	<40	40	
	<i>(2560)</i>	<i>(40)</i>	<i>(&lt;40)</i>	<i>(&lt;40)</i>	
	2	<b>640</b>	<40	40	
	<i>(640)</i>	<i>(40)</i>	<i>(40)</i>	<i>(&lt;40)</i>	
	3	<b>2560</b>	<40	<40	
<i>(2560)</i>	<i>(&lt;40)</i>	<i>(&lt;40)</i>	<i>(&lt;40)</i>		
4	<b>640</b>	<40	<40	<40	
<i>(2560)</i>	<i>(40)</i>	<i>(40)</i>	<i>(&lt;40)</i>		
5	<b>2560</b>	<40	<40	<40	
<i>(2560)</i>	<i>(160)</i>	<i>(&lt;40)</i>	<i>(160)</i>		
<b>HTNV</b> 1	160	<b>2560</b>	<40	40	
	<i>(160)</i>	<i>(2560)</i>	<i>(40)</i>	<i>(&lt;40)</i>	
	2	<b>2560</b>	<40	<40	
	<i>(&lt;40)</i>	<i>(2560)</i>	<i>(40)</i>	<i>(160)</i>	
	3	<40	<b>10240</b>	40	<40
<i>(&lt;40)</i>	<i>(2560)</i>	<i>(&lt;40)</i>	<i>(40)</i>		
4	160	<b>2560</b>	<40	<40	
<i>(40)</i>	<i>(2560)</i>	<i>(40)</i>	<i>(40)</i>		
5	40	<b>640</b>	<40	160	
<i>(&lt;40)</i>	<i>(640)</i>	<i>(&lt;40)</i>	<i>(40)</i>		
<b>PUUV</b> 1	40	160	<b>2560</b>	<40	
	<i>(160)</i>	<i>(40)</i>	<i>(640)</i>	<i>(40)</i>	
	2	<40	<40	<b>2560</b>	<40
	<i>(&lt;40)</i>	<i>(&lt;40)</i>	<i>(2560)</i>	<i>(&lt;40)</i>	
	3	40	<40	<b>2560</b>	160
<i>(&lt;40)</i>	<i>(40)</i>	<i>(640)</i>	<i>(160)</i>		
4	<40	<40	<b>2560</b>	<40	
<i>(&lt;40)</i>	<i>(40)</i>	<i>(2560)</i>	<i>(40)</i>		
5	40	40	<b>2560</b>	<40	
<i>(40)</i>	<i>(&lt;40)</i>	<i>(2560)</i>	<i>(&lt;40)</i>		
<b>SEOV</b> 1	160	<40	<40	<b>2560</b>	
	<i>(40)</i>	<i>(160)</i>	<i>(40)</i>	<i>(10240)</i>	
	2	<40	<40	<40	<b>640</b>
	<i>(40)</i>	<i>(&lt;40)</i>	<i>(&lt;40)</i>	<i>(2560)</i>	
	3	<40	<40	<40	<b>640</b>
<i>(&lt;40)</i>	<i>(40)</i>	<i>(&lt;40)</i>	<i>(640)</i>		
4	<40	<40	<40	<b>2560</b>	
<i>(&lt;40)</i>	<i>(160)</i>	<i>(40)</i>	<i>(2560)</i>		
5	<40	<40	<40	<b>640</b>	
<i>(&lt;40)</i>	<i>(&lt;40)</i>	<i>(&lt;40)</i>	<i>(640)</i>		

Although a quantitative PCR apparatus nowadays is progressively becoming common laboratory equipment, the costs of the RNA isolation from each well and the costs of performing quantitative RT-PCR reactions, makes it unlikely that the RRNT will be useful for

routine screening. The RRNT is more likely to find application in vaccine development or virus discovery. An important guideline for the classification of hantavirus species as proposed by *The International Committee on Taxonomy of Viruses* is an at least 4-fold difference in two-way cross neutralization tests to classify new hantavirus species (Nichol et al., 2005). RRNT can be used easily for characterization of new hantaviruses and for performing the necessary neutralization tests in a relatively short period of time as there is no requirement for specific animal antibodies to detect residual virus when using RRNT.

The primers and probes used in this study are chosen to be as specific as possible for the hantavirus isolates used with the described RRNT. Other studies have published primer and probe sequences in relation to the detection of hantavirus RNA with qRT-PCR (Garin et al., 2001; Aitichou et al., 2005). These primers and probes were constructed for the detection of all strains of a specific hantavirus species, which can be used without problem in an RRNT assay. In summary, we have established a novel neutralization test for hantaviruses based on qRT-PCR, which is an applicable alternative for FRNT.

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**TRUNCATED RECOMBINANT DOBRAVA VIRUS  
NUCLEOCAPSID PROTEINS, INDUCE STRONG, PROTECTIVE  
AND LONG-LASTING IMMUNE RESPONSES IN MICE**

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## 1. SUMMARY

We describe the cloning and expression of Dobrava-Belgrade virus (DOBV) nucleocapsid proteins and a truncated form consisting of the first 118 N-terminal amino acids, and the capacity of these *E. coli* ICONE 200 expressed recombinant proteins (rNp) to induce a protective immune response against DOBV in mice. As an alternative carrier protein, the outer membrane protein A derived from *Klebsiella pneumoniae* (rP40) has been coupled to different rNp constructs. All recombinant proteins were found to be highly immunogenic after 3 immunizations of rNp. The immunizations resulted in the induction of a strong Np-specific IgG response with a predominance of IgG1 over IgG2b and IgG2a, suggesting a mixed Th1/Th2 cell involvement. A specific IgG3 response could not be detected. Mice immunized with recombinant DOBV rNp without rP40 showed lower nucleocapsid-specific antibody responses in comparison with the rP40 conjugated constructs, but all mice were found to be protected against DOBV challenge. Our results indicate that the rNp constructs coupled to rP40, represent promising vaccine candidates.

## 2. INTRODUCTION

Hantaviruses are trisegmented, negative-stranded RNA viruses, belonging to the family *Bunyaviridae* (Schmaljohn et al., 1985). The genome encodes four structural proteins, the L-segment encoding the RNA polymerase, the M-segment encoding two envelope glycoproteins (G1 and G2), and the S-segment encoding the nucleocapsid protein (Np) (Maes et al., 2004a). Hantaviruses give rise to severe human clinical diseases like hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome, but produce persistent, non-pathogenic infections in rodents (Clement et al., 1997; Peters and Khan, 2002). In Europe, Dobrava-Belgrade virus (DOBV) seems to be the most virulent hantavirus. DOBV can bear a fatality rate up to 12%, mainly due to severe hemorrhagic manifestations (Avsic-Zupanc et al., 1992). Two closely related DOBV subspecies are carried by two rodent species, the yellow-necked mouse (*Apodemus flavicollis*) and the striped field mouse (*Apodemus agrarius*) (Klempa et al., 2003). In Southeastern Europe, *Apodemus flavicollis* is the natural reservoir for DOBV (DOBV-Af strain) (Avsic-Zupanc et al., 1995). In the eastern, northeastern and central part of Europe, DOBV is carried by *Apodemus agrarius* (DOBV-Aa strain) (Plyusnin et al., 2001; Klempa et al., 2004). It was only recently that the first pathogenic DOBV-Aa case was molecularly identified in a patient suffering from HFRS

(Klempa et al., 2004). Currently, no specific treatment or prevention is available against the primary viral etiology of HFRS. Several approaches however, have been employed in the development of hantavirus vaccines. Development of inactivated vaccines against HFRS has been well documented in Asia (Cho et al., 2002; Choi et al., 2003). Recombinant vaccinia viruses (Chu et al., 1995), chimeric hepatitis B virus core-derived particles (Koletzki et al., 2000), packaged sindbis virus replicons (Kamrud et al., 1999), recombinant cytomegaloviruses (Rizvanov et al., 2003) and vaccination with naked DNA (Bharadwaj et al., 1999; Koletzki et al., 2001) are recent vaccination strategies.

Several studies have demonstrated the strong antigenicity of the hantavirus Np. High levels of Np-specific antibodies are already detected during the acute phase of HFRS (Niklasson and Kjelsson, 1988; Kallio-Kokko et al., 2001), with a major antigenic domain for the humoral immune response located at the amino-terminus of the hantavirus Np (Elgh et al., 1996). The coding region of the hantavirus Np is relatively conserved and serological cross-reactivity with Np from other hantaviruses is high (Elgh et al., 1998). The protective immunity against hantaviruses is presumably lifelong, since neutralizing antibodies have been detected in patient sera as late as 50 years after Puumala virus infections (Settergren et al., 1991).

In this report we describe the cloning and expression of DOBV-Np and a truncated form consisting of the first 118 N-terminal amino acids in the *E. coli* ICONE 200 (Improved Cell for Over and Non-leaky Expression) strain using the tryptophan promoter controlled pTEX expression vector (Nguyen et al., 1998; Chevalet et al., 2000), and the capacity of these recombinant proteins to induce a strong and protective immune response against DOBV in mice. The *E. coli* ICONE 200-expressed recombinant proteins, carry the whole or the periplasmic part (rP40p) of the *Klebsiella pneumoniae* (rP40) outer membrane protein A, which has been proven to be an excellent adjuvant in mice, and which can potentially be used for human vaccines (Haeuw et al., 1998; Soulas et al., 2000; Libon et al., 2002).

### **3. MATERIALS AND METHODS**

#### ***3.1 Construction, expression and purification of the recombinant proteins.***

The recombinant proteins were cloned and expressed as described in Chapter 4. Briefly, DOBV RNA (strain DOB-90/5) was extracted from infected Vero E6 cells (CRL 1586, ATCC, USA). The genomic RNA of DOBV was reverse-transcribed, and PCR-amplified in order to generate the entire S segment, using following oligonucleotide primers: 5'-GCG AAT

TCG CAA CAC TAG AGG AAC TCC AAA AGG-3' and 5'-CGA AGC TTA GTG GTG GTG GTG GTG AAG TTT GAG CGG CTC C-3'. The amino-terminal part encoding the first 118 amino acids was generated using 5'-CTG GCG CCT AAC CGA CGT GGT GGT GGT GGT GAT TCG AAG C-3' as reverse primer. In all constructs, a histidine (His)-tag was introduced at the C-terminal end. PCR fragments were cloned in plasmids pTEX(rP40) (Nguyen et al., 1998) or pTEXmp18 (Chevalet et al., 2000), respectively with or without the inclusion of the P40 sequence in the construct. Following transformation of the *E. coli* ICONE 200 strain, the recombinant proteins were produced as intracellular inclusion bodies, recovered, and renatured as previously described (Power et al., 1997). The recombinant proteins were purified by metal chelate affinity chromatography using a HisTrap kit (Pharmacia, Puurs, Belgium). Using this protocol, four DOBV rNp constructs were expressed and purified. The complete nucleocapsid protein of DOBV was expressed with or without the addition of the rP40 protein (constructs P40-Dob-N and Dob-N). The amino-terminal part of the DOBV nucleocapsid protein was expressed with the addition of the rP40 protein (construct P40-Dob118) or with the addition of only the periplasmic part of the rP40 protein (construct P40p-Dob118).

### **3.2 Western blotting**

The purified recombinant proteins were subjected to electrophoresis through pre-cast 12% SDS-PAGE Bis-Tris gels (Invitrogen, Leek, The Netherlands). Immunoblotting was performed using DOBV-specific human serum samples (DOBV RNA RT-PCR positive, ELISA IgM and IgG positive).

### **3.3 Animal immunizations**

To assess the immunogenic capacity of the DOBV rNp constructs, 6-week-old outbred NMRI mice (Elevage Janvier, Le Genest Saint Isle, France) were immunized three times subcutaneously with three different concentrations (0.2 µg, 2 µg and 10 µg) of rNp with intervals of two weeks. The animals were injected with Dob-N rNp emulsified in 2% Alhydrogel (Accurate Chemical & Scientific Corp, Westbury, USA). The rNp P40-Dob-N, P40-Dob118 and P40p-Dob118 were administered in sterile PBS. Blood was drawn 14 days after each immunization.

### **3.4 *DOBV-challenge experiments***

To illustrate the protective capacity of the different constructs, groups of 10 NMRI mice were first immunized three times subcutaneously with three different concentrations (0.2 µg, 2 µg and 10 µg) of the different rNp with intervals of two weeks, and were challenged intraperitoneally with  $10^5$  copies of DOBV three weeks after the last immunization. Four weeks later, all mice were sacrificed and serum samples were collected. Mice immunized one, two or three times subcutaneously with different concentrations of rP40 and challenged with DOBV, were used as positive control.

### **3.5 *Detection of Np-specific antibodies***

Mouse sera were analyzed for the presence of rNp-specific antibodies with a solid phase enzyme-linked immunosorbent assay (ELISA). The endpoint titration of the sera was done as previously described (Maes et al., 2004b). Briefly, Maxisorp immunoplates (Nalgene Europe, Neerijse, Belgium) were coated with 5 µg of Dob-118 rNp. Mouse serum samples were diluted in PBS-0.05% Tween-20 in duplex, starting at a dilution of 1:150. After incubation, washed plates were incubated with horseradish-peroxidase conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b and IgG3 antibodies (Southern Biotechnology Associates Inc., Birmingham, USA). After 60 minutes, the plates were washed and incubated with TMB membrane substrate (3,3',5,5'-Tetramethylbenzidine) (Calbiochem, San Diego, USA) for 10 minutes in the dark. Color development was stopped with an equal volume of 1N H<sub>2</sub>SO<sub>4</sub>. Absorbance was read at 450 nm against a reference wavelength of 620 nm. The cut-off value was defined as the mean optical density (OD) plus three standard deviations, calculated with 20 serum samples from mice vaccinated with rP40. The endpoint titer was defined as the serum dilution where the OD was at least equal to the cut-off value.

### **3.6 *Replication reduction neutralization test (RRNT)***

The replication reduction neutralization test (RRNT) was carried out in 96-well plates. All dilutions of sera and viruses were done by using MEM supplemented with 2% heat-inactivated fetal calf serum. Virus ( $2.5 \times 10^2$  hantavirus copies/mL) was mixed with an equal volume of a serum dilution. After a pre-incubation of 1h at 37°C in a humidified 5% CO<sub>2</sub> atmosphere, 20 µl/well of the virus/antibody mixtures were added to a 96-well plate and incubated for 1h at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. For each serum sample tested, four wells were infected with virus incubated with medium (non-neutralized virus) as controls and every serum dilution was run in twofold. After the incubation period, 180 µl of pre-

warmed (37°C) MEM was added to each well and plates were again incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. After an incubation of 10 days, viral RNA was extracted by using the QIAamp viral RNA kit (Qiagen, Leusden, The Netherlands). The titers of infectious virus were quantified by qRT-PCR as described before (Maes et al., 2006). Briefly, qRT-PCR was carried out using the Eurogentec One Step RT qPCR kit (Eurogentec, Seraing, Belgium) with the ABI Prism 7500 Fast Sequence Detection System (Applied Biosystems). The reaction was conducted in a 25 µl volume containing 5 µl of extracted RNA, 12,5 µl of One step RT qPCR MasterMix (Eurogentec, Seraing, Belgium) containing ROX as a passive reference, 900 nM forward and reverse primer, 250 nM FAM-TAMRA probe and 0.125 µl Euroscript/RNase inhibitor (Eurogentec). Reverse transcription was initiated at 48°C for 30 min, followed by PCR activation at 95°C for 10 min and 45 cycles of a two-step incubation at 95°C for 15 s and 60°C for 1 min. The reporter dye (FAM) signal was measured against the internal reference dye (ROX) to normalize for non-PCR-related fluorescence emissions. The threshold cycle (CT) was defined as the fractional cycle number at which the reporter fluorescence, generated by cleavage of the probe, reaches a threshold defined as 10 times the standard deviation of the mean baseline emission.

### ***3.7 Antigen-specific T cell detection by flow cytometric assay for degranulation***

Outbred NMRI mice were immunized once with 10 µg of the different DOBV rNp constructs. The animals were injected with Dob-N rNp emulsified in 2% Alhydrogel. The rNp P40-Dob-N, P40-Dob118 and P40p-Dob118 were administered in sterile PBS. 15 days after immunization, the animals were sacrificed, lymph nodes were removed, and cell suspensions were prepared in RPMI-1640 medium. Stimulation and characterization of the antigen-specific T cells was performed as described elsewhere (Sun et al., 1998; Betts et al., 2003) with some modifications. Briefly, 10<sup>6</sup> cells were incubated with 1 µg each of anti-CD28 and anti-CD49d (BD, Becton Dickinson, San Diego, CA, USA), together with 4 µg/ml lysyl endoproteinase, trypsin and V8-protease digested Dob-N rNp in a 200 µL volume. Staphylococcus enterotoxin B (SEB, 1 µg/ml, Sigma-Aldrich, St. Louis, MO, USA) was used as positive control. Fluorescein isothiocyanate (FITC)-conjugated CD107a and CD107b antibodies (BD) were added to the cells prior to stimulation. In every experiment a negative control (anti-CD28/CD49d) was included to control for spontaneous production of cytokine and/or expression of CD107a/b. The cells were incubated for 1h at 37°C with 5% CO<sub>2</sub>, followed by an additional 5h in the presence of 1 µg/200 µL Brefeldin A and monensin (Sigma-Aldrich). At 6h, 20 µl 20 mM EDTA was added to the cell suspensions and incubated

at room temperature for 15 minutes. Immediately following stimulation, cells were washed once, and surface stained with directly conjugated antibodies (BD, anti-CD3 Pacific Blue, anti-CD4 PerCP, anti-CD8 PE, and anti-CD69 PE-Cy7). The cells were washed and then fix/permeabilized by using the IntraPrep Permeabilization Reagent (Beckman Coulter, Anolis, Erembodegem, Belgium). After Permeabilization, the cells were washed and stained with directly conjugated antibodies specific for intracellular markers (BD, anti-IL2 APC, anti-INF $\gamma$  APC, and anti-TNF $\alpha$  APC). The cells were washed a final time and resuspended in 0.5% paraformaldehyde in PBS. Flow cytometric analysis was performed using a FACSCanto II flow cytometer (BD). List mode data files were analyzed using BD FACSCanto II System Software. In all cases at least 100,000 live events were collected for analysis.

**TABLE 6.1:** The different recombinant forms of the Dobrava virus nucleocapsid protein used in this study.

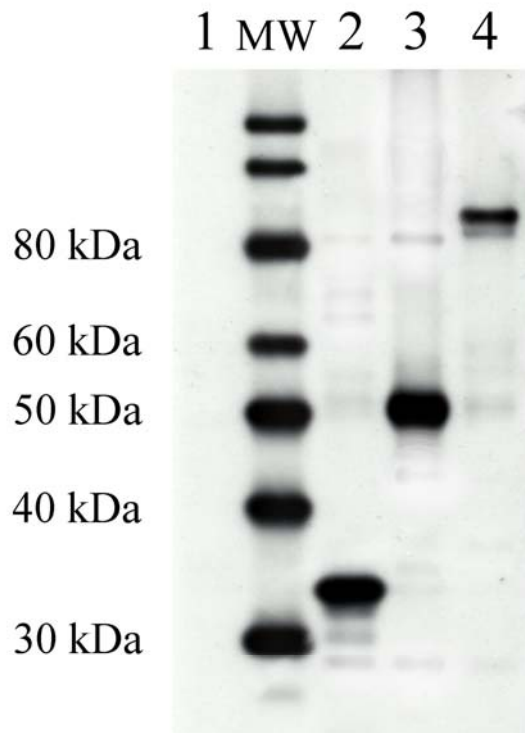
Construct	Molecular weight	
Dob-N	50.1 kDa	The complete nucleocapsid protein.
P40-Dob-N	86.2 kDa	The complete nucleocapsid protein bound to the outer membrane protein A of <i>Klebsiella pneumoniae</i> (rP40).
P40-Dob118	51.1 kDa	The first 118 N-terminal amino acids bound to the outer membrane protein A of <i>Klebsiella pneumoniae</i> (rP40)
P40p-Puu118	30.6 kDa	The first 118 N-terminal amino acids bound to the periplasmic part of the outer membrane protein A of <i>Klebsiella pneumoniae</i> (rP40p).

## 4. RESULTS

### 4.1 Expression and antigenicity of the DOBV rNp proteins

The sequences encoding the Np of DOBV or its truncated forms were correctly cloned in the different pTEX expression vectors as confirmed by complete nucleotide sequencing of the constructs. In order to test the reactivity of the rNp, western blotting was carried out using both DOBV-positive and DOBV-negative human serum samples. With western blotting,

protein bands corresponding to the expected molecular mass (FIGURE 6.1) were revealed for all four rNp (TABLE 6.1). The protein bands showed strong and specific reactions with the DOBV-specific human serum whereas they were not recognized by the DOBV-negative human serum samples.

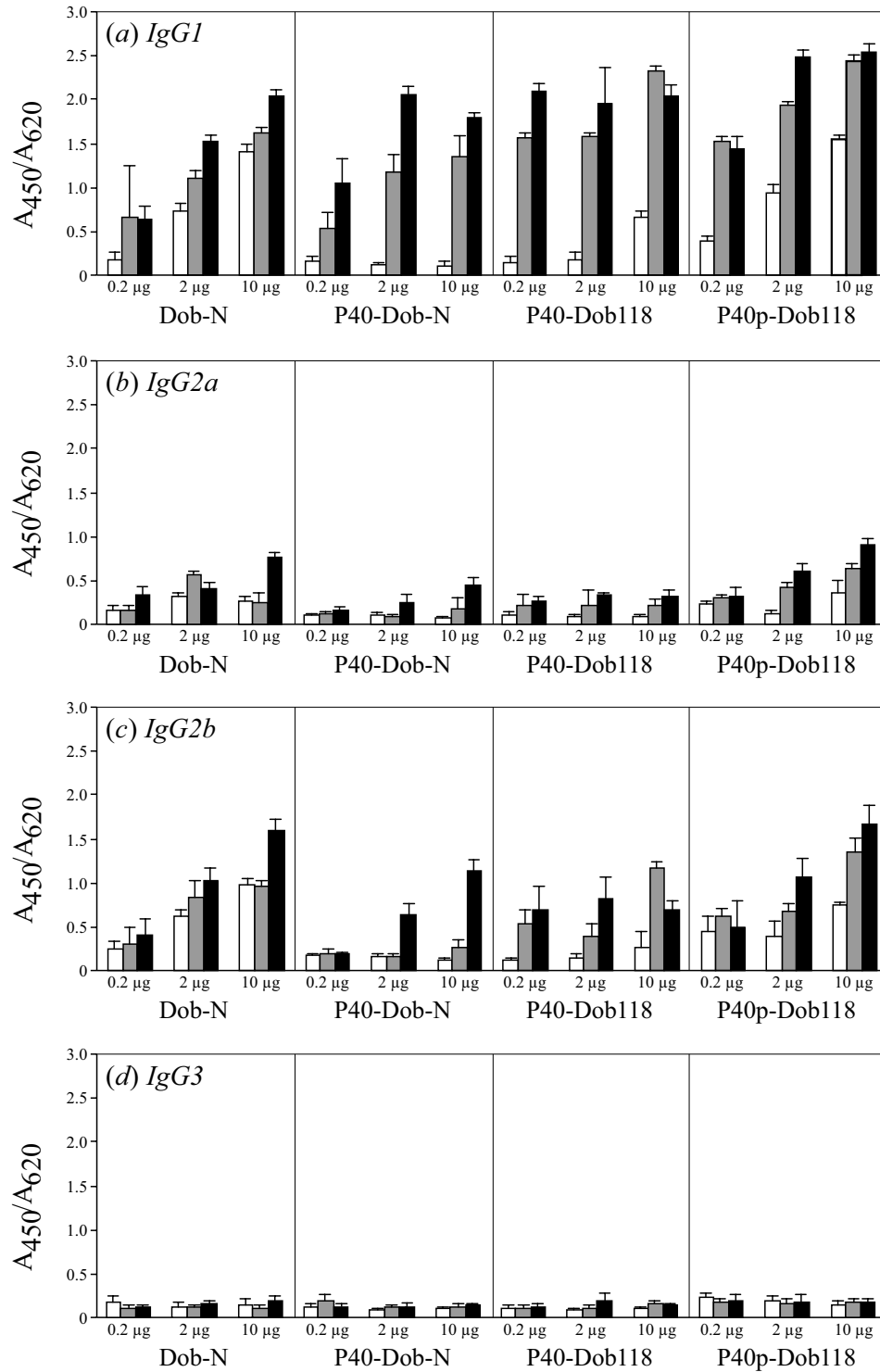


**FIGURE 6.1:** Western blotting analysis of the purified recombinant nucleocapsid proteins: irrelevant His-tagged protein (column 1), P40p-Dob118 (column 2), Dob-N (column 3) and P40-Dob-N (column 4). Blotting was performed using DOBV-positive human serum specimens.

#### ***4.2 Humoral immune responses to DOBV rNp proteins***

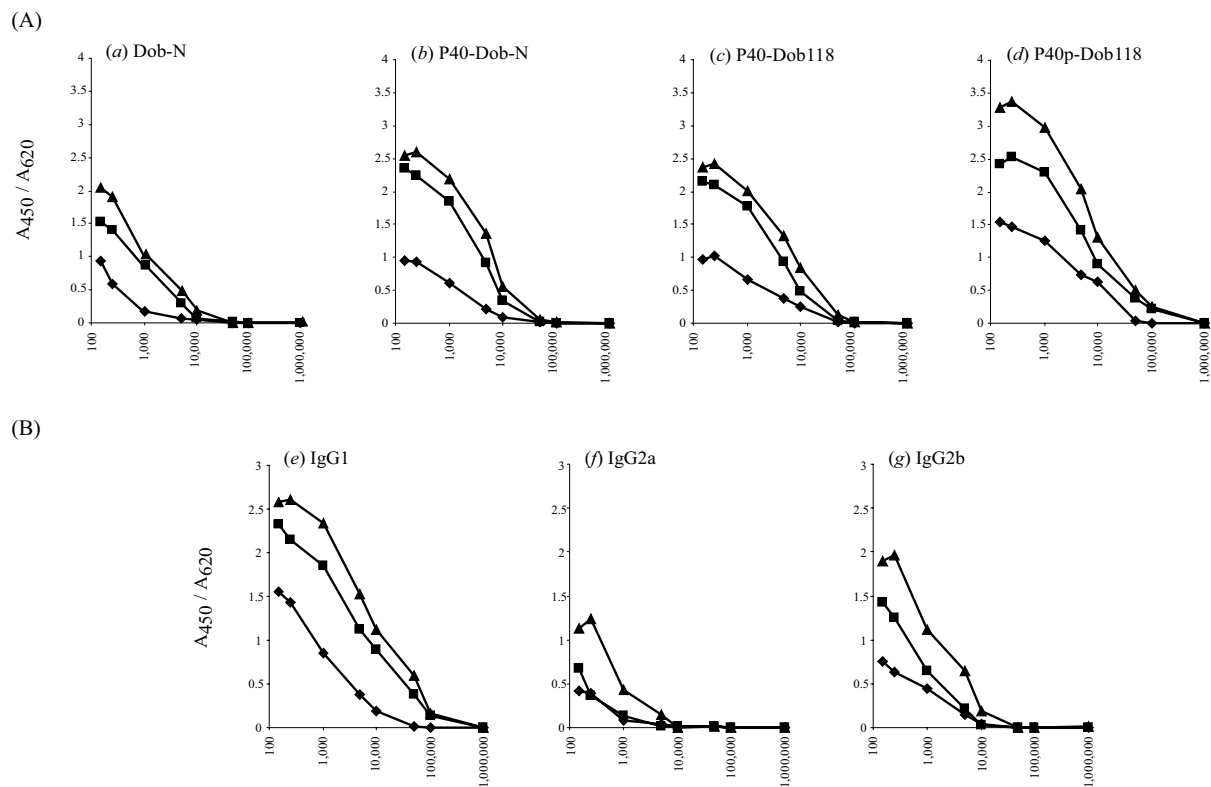
We analyzed the immunogenicity of the four rNp constructs by immunizing NMRI mice up to three times with different rNp concentrations. Individual sera, taken two weeks after each immunization, were tested in ELISA with Dob-N rNp for the presence of DOBV-specific IgG subclass antibodies. As shown in FIGURE 6.2, all four rNp constructs elicited high DOBV-specific antibody responses after three immunizations with 2  $\mu$ g of the proper rNp construct. For the truncated rNp constructs, P40-Dob118 and P40p-Dob118, two immunizations with 0.2  $\mu$ g were found to be sufficient to raise high DOBV-specific antibody responses. The Dob-N rNp construct raised high antibody responses after 3 immunizations with 2  $\mu$ g and 2 immunizations with 10  $\mu$ g. But the overall antibody response generated by Dob-N was lower

than for the truncated rNp constructs. The P40-Dob-N rNp construct raised lower antibody responses in comparison with the other P40-conjugated rNp constructs. DOBV-specific antibodies remained undetectable until the second immunization with the rNp construct.



**FIGURE 6.2:** Dose-response curves from mice immunized one (□), two (■) or three (■) times with 0.2, 2 or 10 µg of the proper rNp, determined by ELISA with Dob-N rNp for (a) IgG1, (b) IgG2a, (c) IgG2B, (d) IgG3.

Endpoint IgG antibody titers were determined for all four rNp constructs (FIGURE 6.3) in pooled sera of mice immunized with 10  $\mu$ g of the proper rNp construct. Two weeks after the first immunization, Np-specific IgG antibody titers were as high as 1:5,000 for Dob-N. For P40-Dob-N, endpoint titers were as high as 1:50,000 after the first immunization. For these rNps, the antibody titers after the second and third immunization raised strongly, but the endpoint titers remained the same (1:5,000 and 1:50,000 for DOB-N and P40-Dob-N respectively). For the truncated rNp constructs, two weeks after the third immunization endpoint antibody titers were as high as 1:100,000 and 1:1,000,000 for P40-Dob118 and P40p-Dob118 respectively. Up to 500 days after the first immunization with 10  $\mu$ g, antibody titers were still as high as 1:1,000,000 for P40p-DOB118. For the other rNps, titers dropped with an average of log 1 after 200 days.

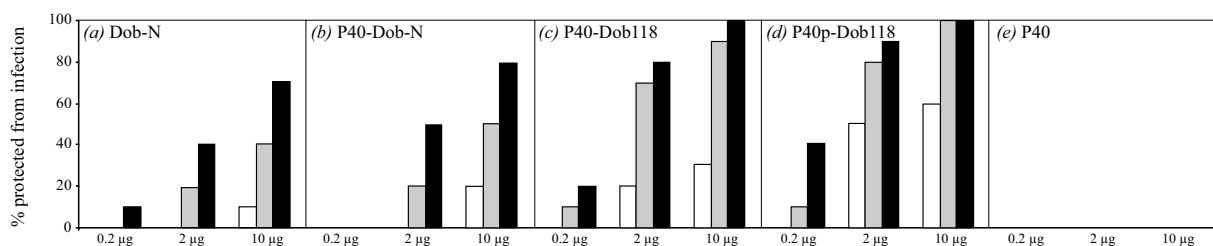


**FIGURE 6.3:** (A) Analysis of the Np-specific IgG antibody response induced in NMRI mice by immunization with 10  $\mu$ g (a) Dob-N, (b) P40-Dob-N, (c) P40-Dob118 and (d) P40p-Dob118. Blood was taken 2 weeks after the first ( $\blacklozenge$ ), second ( $\blacksquare$ ) and third ( $\blacktriangle$ ) immunization. Endpoint titers were determined by ELISA using Dob-N rNp. (B) Identification of the Np-specific IgG subclass antibodies in sera of NMRI mice immunized with P40p-Dob118. Endpoint titers were determined by ELISA with Dob-N rNp for (e) IgG1, (f) IgG2a and (g) IgG2b. No Np-specific IgG3 response could be determined.

In order to elucidate the induced immune responses by the rNp constructs, we determined the IgG subclass specific antibody responses to the rNp constructs. For all four rNp constructs, IgG1 was the predominant subclass over IgG2b and IgG2a and this for each concentration after each immunization (FIGURE 6.2). In none of the sera, a specific IgG3 antibody response could be detected.

#### 4.3 Protection of NMRI mice from infection with DOBV

Mice immunized with different concentration of the rNp constructs were infected intraperitoneal with DOBV to assess the protective capacity of the rNp constructs. During the infection no symptoms in any of the mice were noted. Four weeks after the DOBV infection, mice were sacrificed and serum was tested for the presence of neutralizing antibodies. All mice immunized with rP40 were infected, as shown by high titers of neutralizing antibodies. All mice ( $n = 10$ ) of the group immunized two times with 10  $\mu\text{g}$  P40p-Dob118 were protected against DOBV challenge (100% protection, FIGURE 6.4) as shown by the absence of neutralizing antibodies in the serum of these mice. A single vaccination with 10  $\mu\text{g}$  rNp resulted in a protection of 60%. The group treated three times with 10  $\mu\text{g}$  P40-Dob118 also showed 100% protection. A single and second immunization with 10  $\mu\text{g}$  P40-Dob118 resulted in a protection of respectively 30% and 90%. For the Dob-N and P40-Dob-N immunized groups, three immunizations with 10  $\mu\text{g}$  rNp, resulted in a protection of respectively 70% and 80%. A lesser amount of rNp or fewer immunizations, resulted in protections of 50% or less for both Dob-N and P40-Dob-N (FIGURE 6.4).

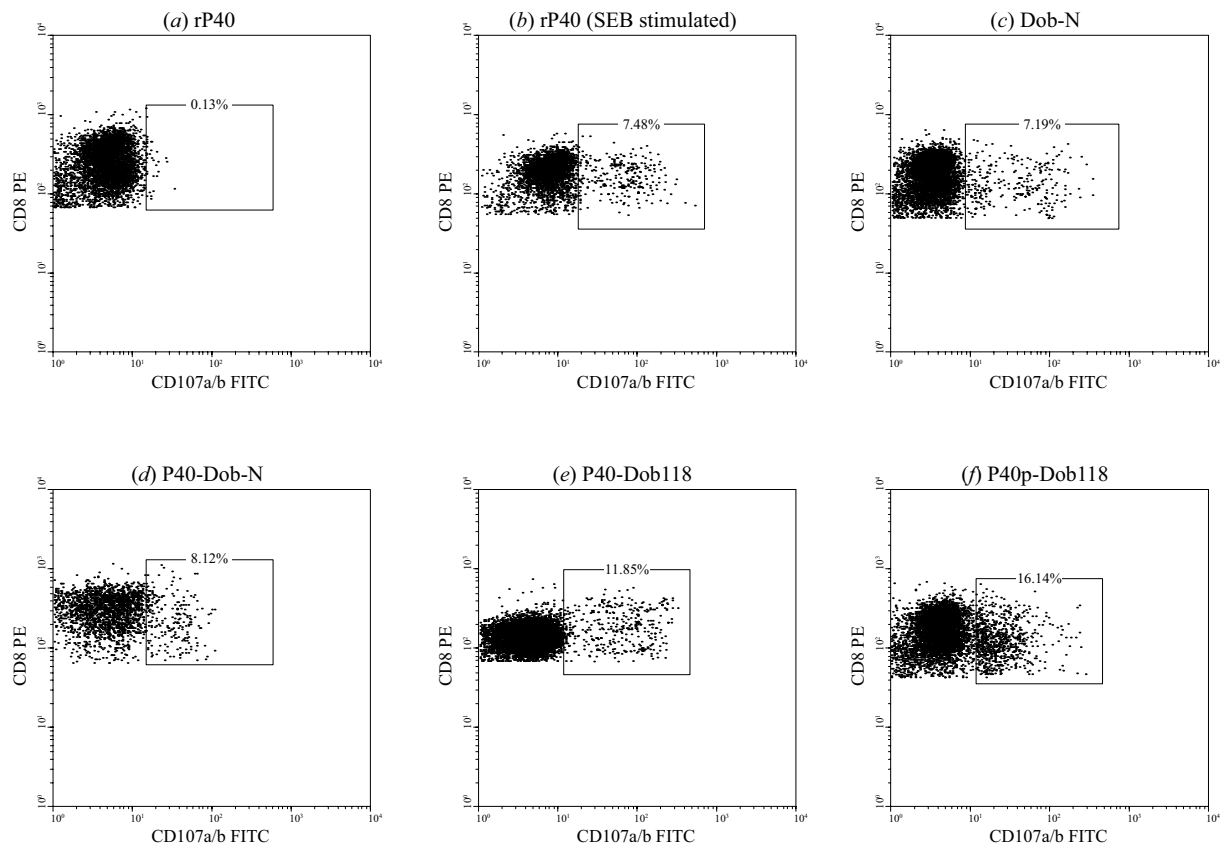


**FIGURE 6.4:** Protection from DOBV challenge after vaccination. NMRI mice were immunized one (□), two (■) or three (■) times with 0.2, 2 or 10  $\mu\text{g}$  of the proper rNp. Mice were sacrificed 4 weeks after challenge and neutralizing antibodies were detected using RRNT. A mouse was considered protected against challenge if no neutralizing antibodies were detected. Mice immunized with rP40 and challenged with DOBV, were used as positive controls.

#### 4.4 Antigen-specific T-cell responses to DOBV rNp proteins

The immunogenicity of the rNp proteins was further evaluated at the T-cell level. PBMC isolated out of lymph nodes of immunized NMRI mice, were stimulated with Dob-N peptides *ex vivo*, and antigen-specific CD8<sup>+</sup> T lymphocytes (cytotoxic T lymphocytes, CTL) were identified by flow cytometry. Efficient CTL activation was achieved in all four groups after a single immunization with 10 µg of the different rNp, whereas no CTL were activated in the control group, NMRI mice immunized with 10 µg rP40 (FIGURE 6.5). The P40p-Dob118 protein demonstrated the highest DOBV-specific T lymphocytes

For the P40-Dob118-N, the P40-Dob-N and the Dob-N immunized NMRI mice, respectively 8.91% and 11.56% of the CD8<sup>+</sup> T lymphocytes were specific for the PUUV Np. For the NMRI mice immunized once with 10 µg Puu-N, 6.68% of the CTL were specific for DOBV Np. These results resemble the variations observed in the challenge experiments.



**FIGURE 6.5:** Characterization of antigen-specific CD8<sup>+</sup> T cells. Pooled PBMC from NMRI mice immunized once with (c) Dob-N, (d) P40-Dob-N, (e) P40-Dob118, and (f) P40p-Dob118, were stimulated *ex vivo* with Puu-N peptides and incubated with antibodies to CD107a and b FITC in the presence of Brefeldin A and monensin, then stained with CD3, CD4, and CD8. (a) NMRI mice immunized once with rP40 were used as negative control. (b) Pooled PBMC from rP40 immunized NMRI mice and stimulated *ex vivo* with SEB were used as positive control. Events shown are gated for CD3 and CD8.

## 5. DISCUSSION

DOBV causes severe hemorrhagic fever with renal syndrome in the Balkan region and elsewhere in Europe and Russia. Since there is no specific treatment or prevention available for DOBV and other hantaviruses, we cloned and expressed different DOBV rNp constructs in the *E. coli* ICONE 200 strain and evaluated these constructs in NMRI mice as possible vaccine candidates. Several research groups have used yeast-expressed hantavirus rNp constructs to evaluate its immunogenicity and use in hantavirus prevention (Dargeviciute et al., 2002; de Carvalho et al., 2002; Geldmacher et al., 2004). A major disadvantage of these described methods is the applicability in human vaccination. For this reason rP40, the recombinant outer membrane protein A of *Klebsiella pneumoniae*, was included in our DOBV rNp constructs. The rP40 adjuvant, which can possibly be used in human vaccines [Centre d'Immunologie Pierre Fabre, personal communication], induces a mixed IFN $\gamma$ -secreting Th1 cell and IL4-secreting Th2 cell response [(Raully et al., 1999), Centre d'Immunologie Pierre Fabre, personal communication]. In our experiments, mice vaccinated with the rP40 conjugated rNp constructs, elicited higher antibody titers than mice vaccinated with the Dob-N rNp construct emulsified in Alhydrogel. Moreover, a higher Np-specific antibody response was observed, despite pre-existing anti-rP40 antibodies generated by multiple preimmunizations with the rP40 molecule (data not shown). This observation, together with the fact that no conventional adjuvant is required, makes the use of rP40 a particularly practical immunopreventive approach.

Immunization of NMRI mice resulted in the induction of a strong Np-specific IgG response with a predominance of IgG1 over IgG2b and IgG2a, suggesting a mixed Th1/Th2 cell involvement. Pooled sera of NMRI mice immunized with P40p-Dob118 rNp showed endpoint antibody titers of 1:1.000.000 up to 500 days after the initial immunization, whereas for Dob-N endpoint antibody titers dropped to 1:250 after 500 days, suggesting a strong induction of memory B cells by rP40p.

In contrast to our study, Geldmacher and colleagues (Geldmacher et al., 2004) detected Np-specific antibodies of all IgG subclasses (IgG1, IgG2a, IgG2b and IgG3) in C57BL/6 mice immunized with recombinant Np of the DOBV Slovenia strain, whereas we detected only Np-specific antibodies of the IgG subclasses IgG1, IgG2a and IgG2b, but not of the IgG3 subclass. Whether this difference to our data might be due to the use of different adjuvants or due to different DOBV strains, remains to be elucidated.

Mice immunized two or three times subcutaneously with 10 µg of the P40p-Dob118 rNp construct were fully protected against challenge with DOBV. As DOBV is apathogenic for mice, neutralizing antibodies were measured with RRNT four weeks after challenge to evaluate the infection. Of the control group immunized with rP40, all became infected as shown by the presence of neutralizing antibodies. None of the mice immunized two or three times with 10 µg P40p-Dob118 rNp construct were infected as no neutralizing antibodies could be detected. Partial protection after challenge with DOBV has been reported before, in C57BL/6 mice immunized with recombinant Np derived from DOBV Slovenia strain and emulsified in Freund's adjuvant (Klingstrom et al., 2004). C57BL/6 mice immunized with rNp in combination with Alum, did not show any specific protection from challenge. In our study, the NMRI mice immunized with Dob-N emulsified in 2% Alhydrogel, showed lower IgG titers in comparison with the groups immunized with rP40 conjugated constructs. Also in the challenge experiments, low protection levels were noticed in mice immunized with the Dob-N rNp construct in combination with Alhydrogel.

For analysis of cellular responses, we identified antigen-specific CD8<sup>+</sup> T lymphocytes by using flow cytometry. With this technique, CD8<sup>+</sup> T lymphocytes that mediate killing can be identified easily. Using this technique, DOBV-specific T lymphocytes could be detected after a single immunization with 10 µg protein for all four rNp. The P40p-Dob118 immunized group showed the highest DOBV-specific CTLs over the P40-Dob118, P40-Dob-N and Dob-N groups which resembles the variations observed in the challenge experiments.

In conclusion, we have shown that immunization with the different DOBV rNp constructs, especially P40-Dob118 and P40p-Dob118, resulted in a high and long-lasting Np-specific IgG response. The rP40 conjugated rNp constructs gave raise to higher antibody titers than Dob-N in combination with Alhydrogel, but in the challenge-experiments, all rNp constructs gave protection against DOBV-infection.

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**TRUNCATED RECOMBINANT PUUMALA VIRUS  
NUCLEOCAPSID PROTEINS PROTECT MICE AGAINST  
CHALLENGE *IN VIVO***

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## 1. SUMMARY

In Europe, Puumala virus and Dobrava virus are the major hantaviruses that cause hemorrhagic fever with renal syndrome in humans. As hantaviruses can cause diseases with high morbidity and mortality, and as to date there is no specific treatment, efforts are concentrated on the development of vaccines. In this study we characterized the immunogenicity of recombinant nucleocapsid proteins of Puumala virus (PUUV) linked to a carrier protein corresponding to the outer membrane protein A from *Klebsiella pneumoniae* (rP40). The rP40 molecule is a novel carrier protein which facilitates exogenous antigen uptake by dendritic cells. We cloned and expressed the recombinant PUUV proteins in the *E. coli* mutant ICONE 200 using the tryptophan promoter controlled pTEXmp18 expression vector. All recombinant PUUV proteins were found to be highly immunogenic in NMRI mice after 3 immunizations with 10 µg of the protein. Only the truncated construct, P40-Puu118, gave high antibody titers after already two vaccinations with 0.2 µg. Likewise in the challenge experiments in NMRI mice, only the truncated construct P40-Puu118 resulted in 100% protection after 3 immunizations with 10 µg. The results suggest that especially P40-Puu118 is a good candidate for a recombinant vaccine against Puumala virus. All recombinant proteins linked to rP40, induced high antibody responses indicating that rP40 is a carrier protein with potential for use in other vaccines.

## 2. INTRODUCTION

Members of the genus *Hantavirus*, family *Bunyaviridae*, are rodent-borne or insectivore-borne viruses and some of them are recognized causes of human hemorrhagic fever with renal syndrome (HFRS, endemic in the Old World) or hantavirus cardiopulmonary syndrome (HPS, endemic in the New World) (Schmaljohn and Hjelle, 1997; Kruger et al., 2001; Maes et al., 2004a). The genome of hantaviruses encodes four structural proteins, the L segment encoding the RNA polymerase, the M segment encoding two envelope glycoproteins (G1 and G2), and the S segment encoding the nucleocapsid protein (Np) (Maes et al., 2004a). These viruses have habitually a single principal reservoir host and are supposed to have coevolved with their hosts (Clement et al., 1997). Once infected, the natural hosts of these viruses are persistently infected presumably for life without any sign of likely disease (Yanagihara et al., 1985). Puumala virus (PUUV), carried by *Myodes glareolus* or bank vole, is the causative agent of nephropathia epidemica (NE), a mild form of HFRS that occurs in western and central

Europe, and in western Russia. (Linderholm and Elgh, 2001; Clement et al., 2006). Clinical symptoms include fever, thrombocytopenia, several degrees of renal failure and, in severe cases, shock and hemorrhage (Kruger et al., 2001). Several studies identified the PUUV nucleocapsid protein (Np) as a major antigenic target in early serological responses and demonstrated that high levels of PUUV-Np specific antibodies are produced during the acute phase of the disease (Niklasson and Kjelsson, 1988; Vapalahti et al., 1995; Lundkvist et al., 1996). The coding region of Np is relatively conserved and serological cross-reactivity between Np from other hantaviruses of the same genogroup is high (Elgh et al., 1998). The major antigenic domain for the humoral response to Np is located at the amino-terminus (Elgh et al., 1996). Neutralizing antibodies are elicited by the envelope glycoproteins G1 and G2, and the protective immunity against PUUV is presumably lifelong, since these neutralizing antibodies have been detected in patient sera as late as 50 years after PUUV infections (Settergren et al., 1991).

Currently, no specific treatment or prevention is available against the primary viral etiology of HFRS or HPS except for early and I.V. doses of the nucleoside analogue ribavirin in the setting of epidemics of severe HFRS caused by the Asian prototype virus Hantaan virus (HTNV) in China (Huggins et al., 1991). Several approaches however, have been employed in the development of hantavirus vaccines. Development of inactivated vaccines against HFRS has been well documented in Asia (Cho et al., 2002; Choi et al., 2003). Recombinant vaccinia viruses (Chu et al., 1995), chimeric hepatitis B virus core-derived particles (Ulrich et al., 1998; Koletzki et al., 2000), packaged Sindbis virus replicons (Kamrud et al., 1999), recombinant cytomegaloviruses (Rizvanov et al., 2003) and vaccination with naked DNA (Hooper et al., 1999; Bharadwaj et al., 1999; Koletzki et al., 2001) are recent vaccination strategies.

In this report we describe the cloning and expression of PUUV-Np and a truncated form consisting of the first 118 N-terminal amino acids of the nucleocapsid protein (Table 1) in the *E. coli* mutant ICONE 200 (Improved Cell for Over and Non-leaky Expression) using the tryptophan promoter controlled pTEX expression vector {Chevalet, Robert, et al. 2000 179 /id}(Nguyen et al., 1998), and the capacity of these recombinant proteins to induce a strong and protective immune response against PUUV in outbred NMRI mice. There is no animal model that mimics the human disease caused by PUUV. Although several species of rodents, rabbits and non-human primates can be infected with PUUV, only humans have been shown to present symptoms of HFRS. Several non-lethal rodent models have been described as models for PUUV and Dobrava virus, including models using C57BL/6, outbred NMRI or

*Myodes glareolus* (Dargeviciute et al., 2002; Klingstrom et al., 2004; Maes et al., 2006a). Infection in these rodent models is scored by the presence of neutralizing antibodies. The hantavirus RNA is encapsidated by the nucleocapsid protein. Moreover, this protein is not present on the surface of hantavirus particles. Probably because of this, no neutralizing antibodies targeting the nucleocapsid protein can be detected in infected humans or rodents. Consequently, the C57BL/6 model or the outbred NMRI model, are good models to evaluate possible vaccine candidate derived from the nucleocapsid protein by using neutralizing antibodies as a marker for infection.

The *E. coli* ICONE 200-expressed recombinant proteins, carry the outer membrane protein A (rP40) of *Klebsiella pneumoniae* (an Enterobacteria responsible for respiratory tract and urinary infections). This rP40 molecule is a new type of pathogen-associated molecular pattern (PAMP) and interacts with dendritic cells and macrophages, suggesting that the immune system has acquired the ability to recognize this type of PAMP. In addition to activating antigen presenting cells (APC), rP40 favors antigen internalization and cross-presentation by APCs. Thus, rP40 is a new protein vector which is able to trigger the initiation of protective anti-virus cytotoxic responses in the absence of CD4 T cell help and adjuvant. This rP40 vector can be used as carrier of vaccine peptides and proteins to facilitate exogenous antigen uptake by dendritic cells (Haeuw et al., 1998; Soulas et al., 2000; Libon et al., 2002; Jeannin et al., 2005; Maes et al., 2006a).

### **3. MATERIALS AND METHODS**

#### **3.1 *Virus and cell culture***

Vero E6 cells (American Type Culture Collection, C1008) were cultured in minimum essential medium (MEM) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum. The maintenance medium for virus propagation was identical but contained 2% (vol/vol) fetal calf serum. The PUUV strain CG1820 (Tkachenko et al., 1984) was propagated for 10 days on monolayers of Vero E6 cells. Cells and virus were cultured at 37°C with 5% CO<sub>2</sub>. PUUV stocks used in the challenge experiments were quantified by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) as previously described (Maes et al., 2006b).

### **3.2 Construction, expression and purification of the recombinant proteins**

The recombinant proteins were cloned and expressed as previously described (Maes et al., 2004b; Maes et al., 2006a). Briefly, PUUV RNA was extracted from infected Vero E6. The genomic RNA of PUUV was reverse-transcribed, and PCR-amplified in order to generate the entire S segment, using following oligonucleotide primers: 5'-CGG AAT TCA GTG ACT TGA CAG ACA TCC AAG-3' and 5'-CGA AGC TTA GTG GTG GTG GTG GTG GTG TAT CTT TAA GGG CTC CTG ATT TG-3'. The N-terminal part encoding the first 118 amino acids was generated using 5'-GGT TCA CCG GTC TGT CGT CTA ACC GTG GTG GTG GTG GTG ATT CGA AGC-3 ' as reverse primer. In all constructs, a histidine-tag was introduced at the C-terminal end. PCR fragments were cloned in plasmids pTEX(rp40) (Nguyen et al., 1998) or pTEXmp18 (Chevalet et al., 2000), respectively with or without the inclusion of the rp40 sequence in the construct. The generated constructions were sequenced using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). DNA sequencing was performed with the Dye-terminator Rhodamine sequencing reaction mix (Applied Biosystems) and DNA sequences were analyzed using Sequencer 3.0 software (Applied Biosystems). Following transformation of the *E. coli* mutant ICONE 200, the recombinant proteins were produced as intracellular inclusion bodies, recovered, and renatured as previously described (Power et al., 1997). The recombinant proteins were purified by metal chelate affinity chromatography using a HisTrap kit (Pharmacia, Puurs, Belgium). Using this protocol, three PUUV rNp constructs were expressed and purified. The complete nucleocapsid protein of PUUV was expressed with or without the addition of the rp40 protein (constructs P40-Puu-N and Puu-N). The N-terminal part of the PUUV nucleocapsid protein was expressed with the addition of the rp40 protein (construct P40-Puu118) (Table 7.1).

### **3.3 Animal immunizations**

To assess the immunogenic capacity of the PUUV rNp constructs, groups of ten 6-week-old outbred male NMRI mice (Elevage Janvier, Le Genest Saint Isle, France) were immunized three times subcutaneously with three different concentrations (0.2 µg, 2 µg and 10 µg) of rNp with intervals of two weeks. The animals were injected with Puu-N rNp emulsified in 2% Alhydrogel (Accurate Chemical & Scientific Corp, Westbury, USA). The rNp P40-Puu-N and P40-Puu118 were administered in sterile PBS. Blood was drawn 14 days after each immunization. The animals were maintained in an approved facility under conditions that met all requirements for animal use.

**TABLE 7.1:** The different recombinant forms of the Puumala virus nucleocapsid protein used in this study.

<b>Construct</b>	<b>Molecular weight</b>	
Puu-N	51.5 kDa	The complete nucleocapsid protein.
P40-Puu-N	87.7 kDa	The complete nucleocapsid protein bound to the outer membrane protein A of <i>Klebsiella pneumoniae</i> (rP40).
P40-Puu118	52.3 kDa	The first 118 N-terminal amino acids bound to the outer membrane protein A of <i>Klebsiella pneumoniae</i> (rP40).

### 3.4 PUUV-challenge experiments

To illustrate the protective capacity of the different constructs, groups of 10 NMRI mice were first immunized three times subcutaneously with three different concentrations (0.2 µg, 2 µg and 10 µg) of the different rNp with intervals of two weeks, and were challenged intraperitoneally with  $10^3$  copies of PUUV three weeks after the last immunization. Animals were sacrificed 28 days post-challenge, and lungs were excised and examined for the presence of PUUV S segment RNA by quantitative RT-PCR (qRT-PCR) as described before (Maes et al., 2006b). Mice immunized one, two or three times subcutaneously with different concentrations of rP40 and challenged with PUUV, were used as positive control.

### 3.5 Detection of Np-specific antibodies

Mouse sera were analyzed for the presence of rNp-specific antibodies with a solid phase enzyme-linked immunosorbent assay (ELISA). The antibody titration of the sera was done as previously described (Maes et al., 2004b). Briefly, Maxisorp immunoplates (Nalgene Europe, Neerijse, Belgium) were coated with 5 µg of Puu118 rNp. Mouse serum samples were diluted in PBS-0.05% Tween-20 in duplex (dilution of 1:400). After incubation, washed plates were incubated with horseradish-peroxidase conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b and IgG3 antibodies (Southern Biotechnology Associates Inc., Birmingham, USA). After 60 minutes, the plates were washed and incubated with TMB membrane substrate (3,3',5,5'-Tetramethylbenzidine) (Calbiochem, San Diego, USA) for 10 minutes in the dark. Color development was stopped with an equal volume of 1N H<sub>2</sub>SO<sub>4</sub>. Absorbance was read at 450

nm against a reference wavelength of 620 nm. The cut-off value was defined as the mean optical density (OD) plus three standard deviations, calculated with 20 serum samples from mice vaccinated with rP40. The endpoint titer was defined as the serum dilution where the OD was at least equal to the cut-off value.

### **3.6 Replication reduction neutralization test (RRNT)**

The replication reduction neutralization test (RRNT) was carried out in 96-well plates. All dilutions of sera and viruses were done by using MEM supplemented with 2% heat-inactivated fetal calf serum. Virus ( $2.5 \times 10^2$  hantavirus copies/mL) was mixed with an equal volume of a serum dilution. After a pre-incubation of 1h at 37°C in a humidified 5% CO<sub>2</sub> atmosphere, 20 µl/well of the virus/antibody mixtures were added to a 96-well plate containing confluent Vero E6 cell monolayers and incubated for 1h at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. For each serum sample tested, four wells were infected with virus incubated with medium (non-neutralized virus) as controls and every serum dilution was run in twofold. After the incubation period, 180 µl of pre-warmed (37°C) MEM was added to each well and plates were again incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. After an incubation of 10 days, viral RNA was extracted by using the QIAamp viral RNA kit (Qiagen, Leusden, The Netherlands). The titers of infectious virus were quantified by qRT-PCR as described before (Maes et al., 2006b). Briefly, qRT-PCR was carried out using the Eurogentec One Step RT qPCR kit (Eurogentec, Seraing, Belgium) with the ABI Prism 7500 Fast Sequence Detection System (Applied Biosystems). The reaction was conducted in a 25 µl volume containing 5 µl of extracted RNA, 12,5 µl of One step RT qPCR MasterMix (Eurogentec, Seraing, Belgium) containing ROX as a passive reference, 900 nM forward and reverse primer, 250 nM FAM-TAMRA probe and 0.125 µl Euroscript/RNase inhibitor (Eurogentec). Reverse transcription was initiated at 48°C for 30 min, followed by PCR activation at 95°C for 10 min and 45 cycles of a two-step incubation at 95°C for 15 s and 60°C for 1 min. The reporter dye (FAM) signal was measured against the internal reference dye (ROX) to normalize for non-PCR-related fluorescence emissions. The threshold cycle ( $C_T$ ) was defined as the fractional cycle number at which the reporter fluorescence, generated by cleavage of the probe, reaches a threshold defined as 10 times the standard deviation of the mean baseline emission.

### ***3.7 Antigen-specific T cell detection by flow cytometric assay for degranulation***

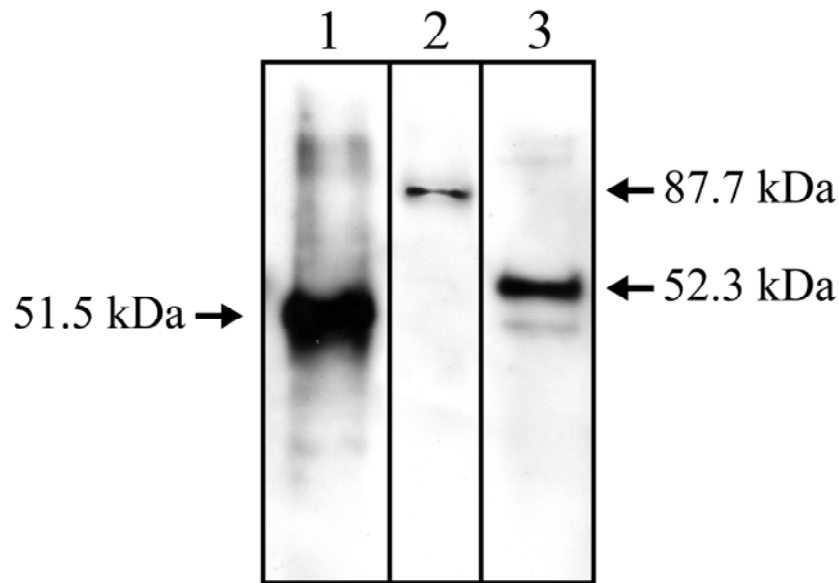
Outbred NMRI mice were immunized once with 10 µg of the different PUUV rNp constructs. The animals were injected with Puu-N rNp emulsified in 2% Alhydrogel. The rNp P40-Puu-N and P40-Puu118 were administered in sterile PBS. 15 days after immunization, the animals were sacrificed, lymph nodes were removed, and cell suspensions were prepared in RPMI-1640 medium. Stimulation and characterization of the antigen-specific T cells was performed as described elsewhere (Suni et al., 1998; Betts et al., 2003) with some modifications. Briefly, 10<sup>6</sup> cells were incubated with 1 µg each of anti-CD28 and anti-CD49d (BD, Becton Dickinson, San Diego, CA, USA), together with 4 µg/ml lysyl endoproteinase, trypsin and V8-protease digested Puu-N rNp in a 200 µL volume. Staphylococcus enterotoxin B (SEB, 1 µg/ml, Sigma-Aldrich, St. Louis, MO, USA) was used as positive control. Fluorescein isothiocyanate (FITC)-conjugated CD107a and CD107b antibodies (BD) were added to the cells prior to stimulation. In every experiment a negative control (anti-CD28/CD49d) was included to control for spontaneous production of cytokine and/or expression of CD107a/b. The cells were incubated for 1h at 37°C with 5% CO<sub>2</sub>, followed by an additional 5h in the presence of 1 µg/200 µL Brefeldin A and monensin (Sigma-Aldrich). At 6h, 20 µl 20 mM EDTA was added to the cell suspensions and incubated at room temperature for 15 minutes. Immediately following stimulation, cells were washed once, and surface stained with directly conjugated antibodies (BD, anti-CD3 Pacific Blue, anti-CD4 PerCP, anti-CD8 PE, and anti-CD69 PE-Cy7). The cells were washed and then fix/permeabilized by using the IntraPrep Permeabilization Reagent (Beckman Coulter, Analis, Erembodegem, Belgium). After Permeabilization, the cells were washed and stained with directly conjugated antibodies specific for intracellular markers (BD, anti-IL2 APC, anti-INF $\gamma$  APC, and anti-TNF $\alpha$  APC). The cells were washed a final time and resuspended in 0.5% paraformaldehyde in PBS. Flow cytometric analysis was performed using a FACSCanto II flow cytometer (BD). List mode data files were analyzed using BD FACSCanto II System Software. In all cases at least 100,000 live events were collected for analysis.

## **4. RESULTS**

### ***4.1 Expression and antigenicity of the PUUV rNp proteins***

The sequences encoding the Np of PUUV or its truncated form were correctly cloned in the different pTEX expression vectors as confirmed by complete nucleotide sequencing of the

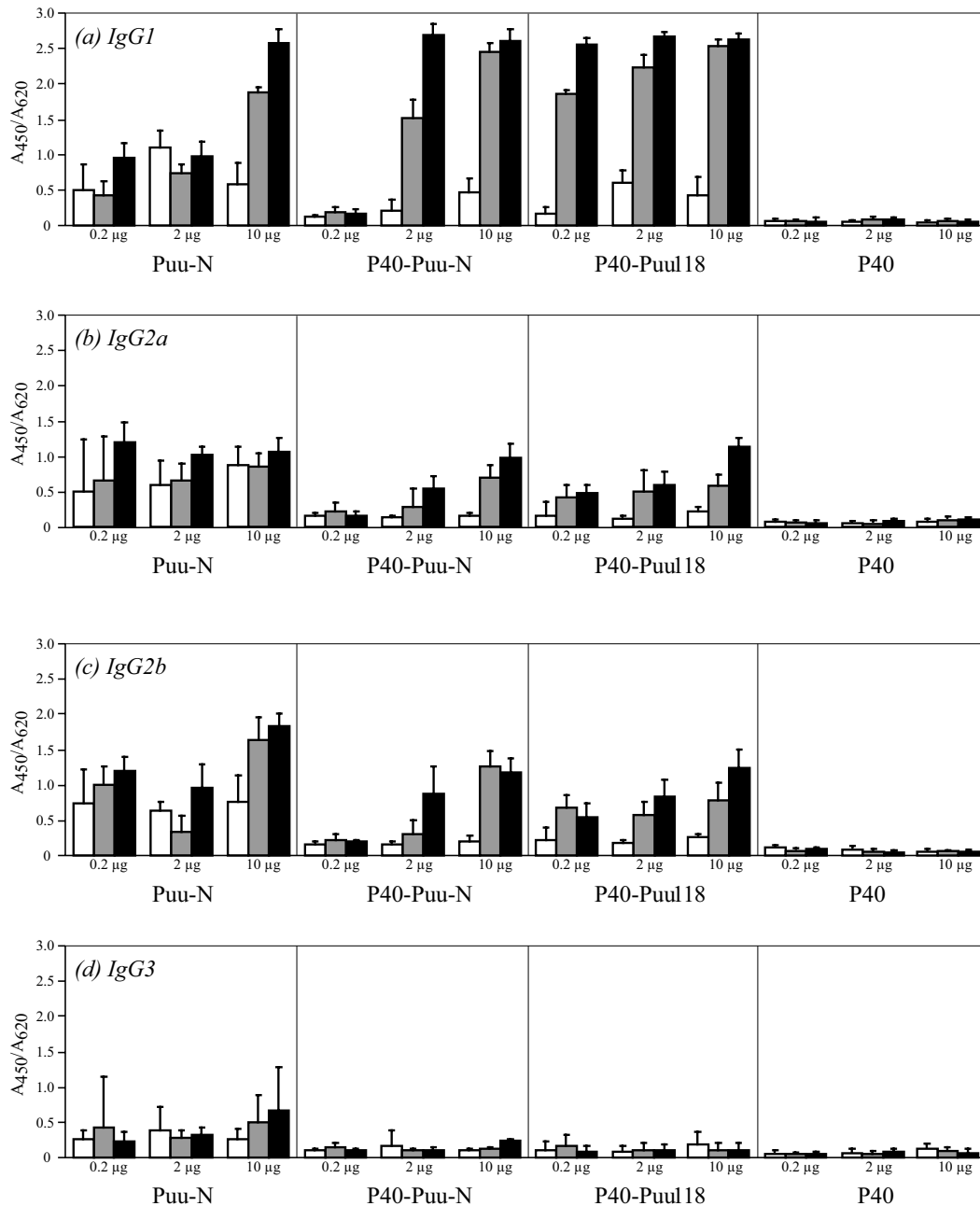
constructs. In order to test the reactivity of the rNps, western blotting was performed by using both PUUV-positive and PUUV-negative human serum samples, and an ECL chemiluminescence detection kit. Protein bands corresponding to the expected molecular mass (TABLE 7.1) were revealed for all three rNp (FIGURE 7.1). The protein bands showed strong and specific reactions with the PUUV-specific human serum whereas they were not recognized by the PUUV-negative human serum samples.



**FIGURE 7.1:** Western blotting analysis of the purified recombinant nucleocapsid proteins: Puu-N (column 1), P40-Puu-N (column 2) and P40-Puu118 (column 3). Blotting was performed using PUUV-positive human serum specimens.

#### **4.2 Humoral immune responses to the PUUV rNp proteins**

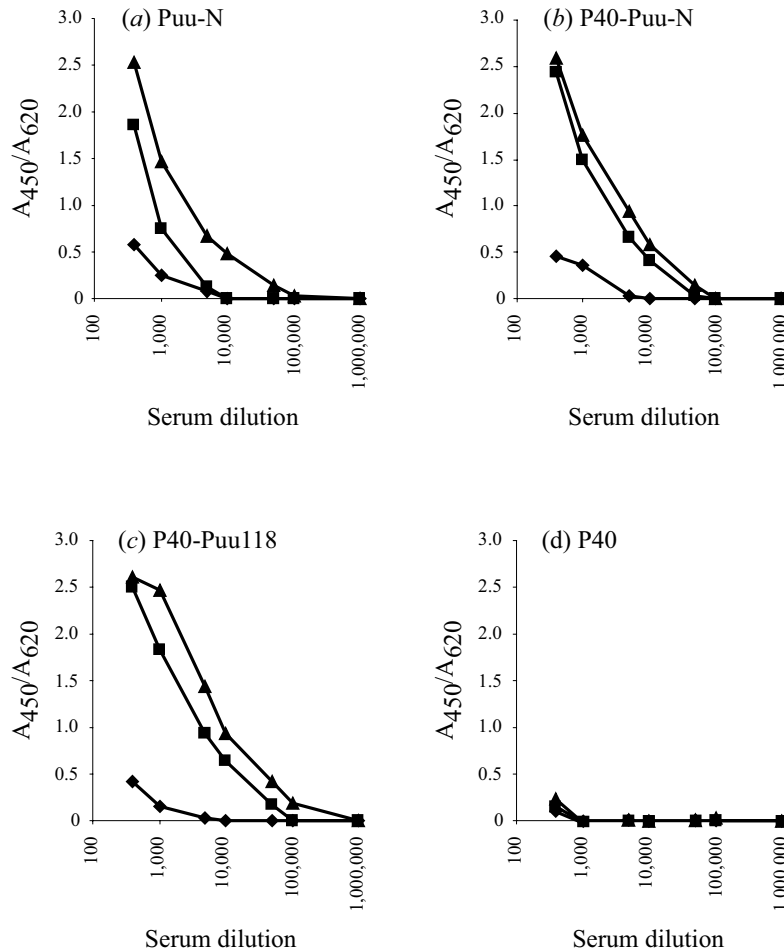
We analyzed the immunogenicity of the three rNp constructs by immunizing NMRI mice up to three times with different rNp concentrations. Individual sera taken two weeks after each immunization were tested with ELISA for the presence of PUUV-specific IgG subclass antibodies. As shown in FIGURE 7.2, all three rNp constructs elicited high PUUV-specific antibody responses after three immunizations with 10  $\mu$ g of the proper rNp construct. For the construct P40-Puu-N two immunizations with 2  $\mu$ g was found to be sufficient to raise high PUUV-specific antibody responses. Moreover, for the truncated P40-Puu118 rNp construct, two immunizations with 0.2  $\mu$ g raised already high PUUV-specific antibody titers. For all three rNp constructs, IgG1 was the predominant subclass over IgG2b and IgG2a and this for each concentration after each immunization. In none of the sera, a specific IgG3 antibody response could be detected.



**FIGURE 7.2:** Dose-response curves from mice immunized one (□), two (■) or three (■) times with 0.2, 2 or 10 µg of the proper rNp, determined by ELISA for (a) IgG1, (b) IgG2a, (c) IgG2B, (d) IgG3. Mice immunized with rP40 using the above described immunization scheme were used as negative control.

Endpoint IgG antibody titers were determined for all three rNp constructs (FIGURE 7.3) in pooled sera of mice immunized with 10 µg of the proper rNp construct. Two weeks after the first immunization, Np-specific IgG antibody titers were as high as 1:5,000 for Puu-N. For P40-Puu-N, endpoint titers were as high as 1:1,000 after the first immunization. For these rNps, the antibody titers after the second and third immunization raised strongly, but the endpoint titers remained relatively low (1:50,000 for both Puu-N and P40-Puu-N). For the

truncated rNp construct P40-Puu118, two weeks after the third immunization endpoint antibody titers were as high as 1:100,000. Up to 300 days after the first immunization with 10  $\mu$ g, antibody titers were still as high as 1:100,000 for this rNp (data not shown).

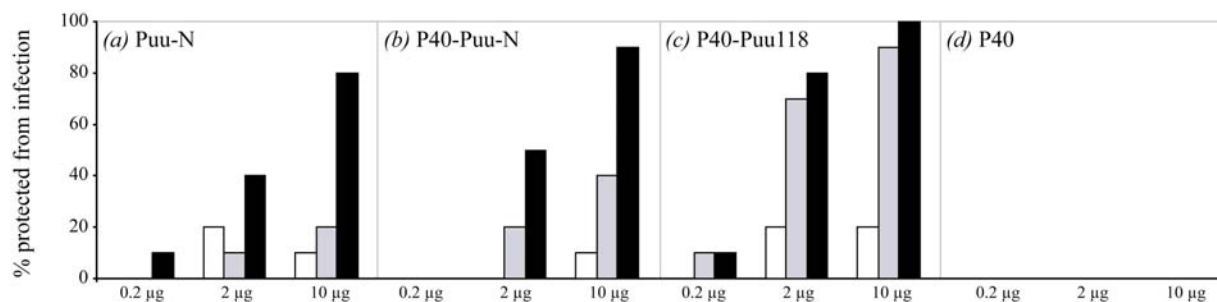


**FIGURE 7.3:** Analysis of the Np-specific IgG antibody response induced in outbred NMRI mice by immunization with 10  $\mu$ g (a) Puu-N, (b) P40-Puu-N, and (c) P40-Puu118. Blood was taken 2 weeks after the first (◆), second (■) and third (▲) immunization. Endpoint titers given in the abscissa were determined by ELISA. Mice immunized one to three times with 10  $\mu$ g of rP40 (d) were used as negative controls.

#### 4.3 Protective efficacy of the PUUV rNp proteins

Mice immunized with different concentrations of the rNp constructs were infected intraperitoneally with PUUV to assess the protective capacity of the rNp constructs. During the challenge experiments no symptoms in any of the mice were noted. Four weeks after virus challenge, mice were sacrificed and the blood and lungs were tested for the presence of viral S segment RNA. The serum was tested for the presence of neutralizing antibodies derived from the G1 and G2 glycoproteins (M segment). In none of the challenged mice, PUUV S segment RNA could be detected with qRT-PCR in the serum, nor in the lungs. All mice ( $n = 10$ ) of the

group immunized three times with 10  $\mu\text{g}$  P40-Puu118 were protected against PUUV challenge (100% protection, FIGURE 7.4) as shown by the absence of neutralizing antibodies in the serum of these mice. Mouse groups treated once or twice with 10  $\mu\text{g}$  P40-Puu118 showed 20% and 90% protection respectively. A two or three times immunization with 2  $\mu\text{g}$  P40-Puu118 gave a protection of 70% and 80% respectively. For the other rNps Puu-N and P40-Puu-N, three immunizations with 10  $\mu\text{g}$  gave a protection of respectively 80% and 90%. A lesser amount of rNp or fewer vaccinations, resulted in protection of 50% or less for both Puu-N and P40-Puu-N (FIGURE 7.4). All mice immunized with only rP40 were infected, as shown by high reciprocal neutralizing endpoint titers, but no viral RNA could be detected in the serum. In only 2 mice ( $n = 10$ ) of this control group, viral S segment RNA could be detected in the lungs demonstrating that, in contrast to the absence of neutralizing antibodies, the absence of hantavirus RNA is not a suitable marker to determine protection in this outbred NMRI model.

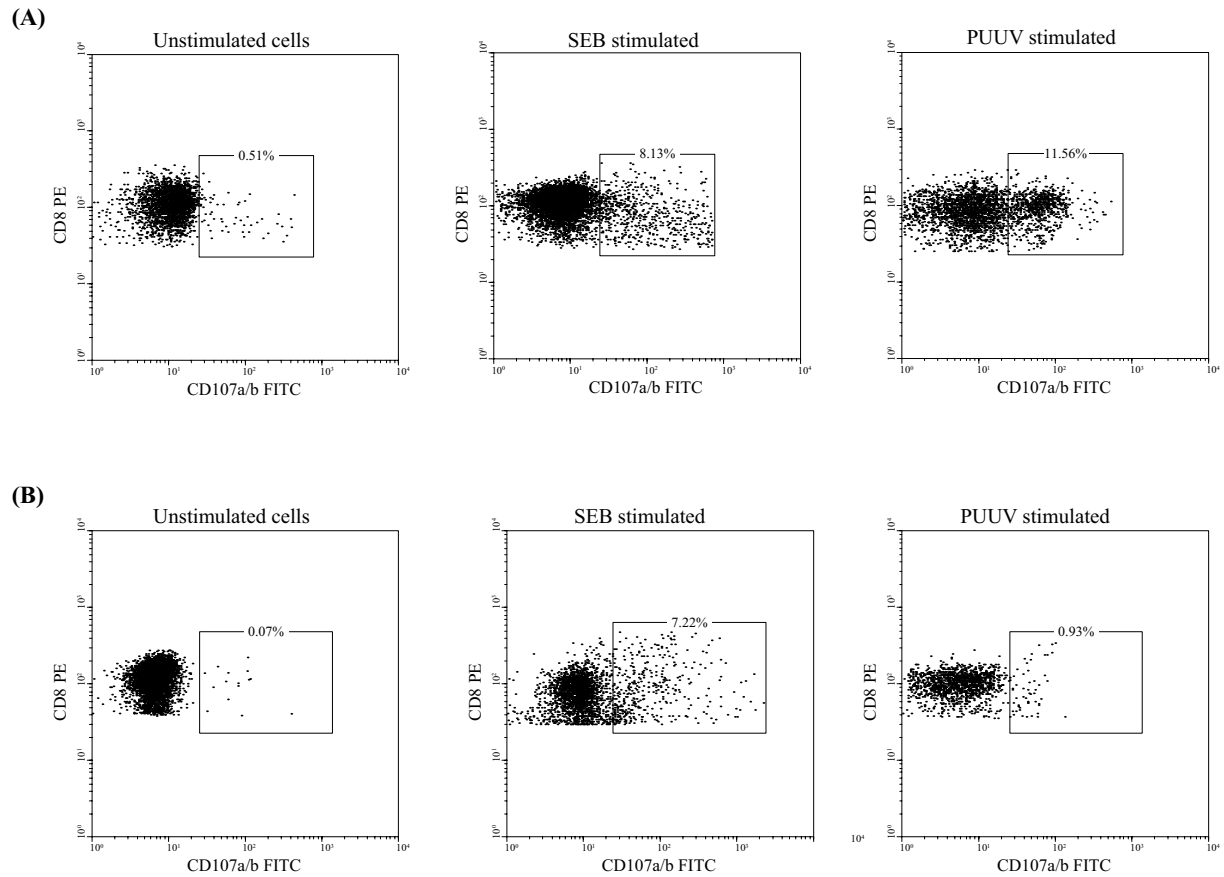


**FIGURE 7.4:** Percent protection from PUUV challenge after vaccination. Groups of 10 NMRI mice were immunized one (□), two (■) or three (■) times with 0.2, 2 or 10  $\mu\text{g}$  of the proper rNp. Mice were sacrificed 4 weeks after challenge and neutralizing antibodies were detected using RRNT. A mouse was considered protected against challenge if no neutralizing antibodies against the G1 and G2 glycoproteins (M segment) were detected. Mice immunized with rP40 and challenged with PUUV, were used as positive controls.

#### 4.4 Antigen-specific T-cell responses to the PUUV rNp proteins

The immunogenicity of the rNp proteins was further evaluated at the T-cell level. PBMC isolated out of lymph nodes of immunized NMRI mice, were stimulated with Puu-N peptides *ex vivo*, and antigen-specific CD8<sup>+</sup> T lymphocytes (cytotoxic T lymphocytes, CTL) were identified by flow cytometry. Efficient CTL activation was achieved in all three groups after a single immunization with 10  $\mu\text{g}$  of the different rNp, whereas no CTL were activated in the control group, NMRI mice immunized with 10  $\mu\text{g}$  rP40 (FIGURE 7.5). For the P40-Puu-N and P40-Puu118 immunized NMRI mice, respectively 8.91% and 11.56% of the CD8<sup>+</sup> T lymphocytes were specific for the PUUV Np. These activated T lymphocytes were highly

positive for IFN $\gamma$  (FIGURE 7.6). For the NMRI mice immunized once with 10  $\mu$ g Puu-N, 6.68% of the CTL were specific for PUUV Np. These results resemble the variations observed in the challenge experiments.

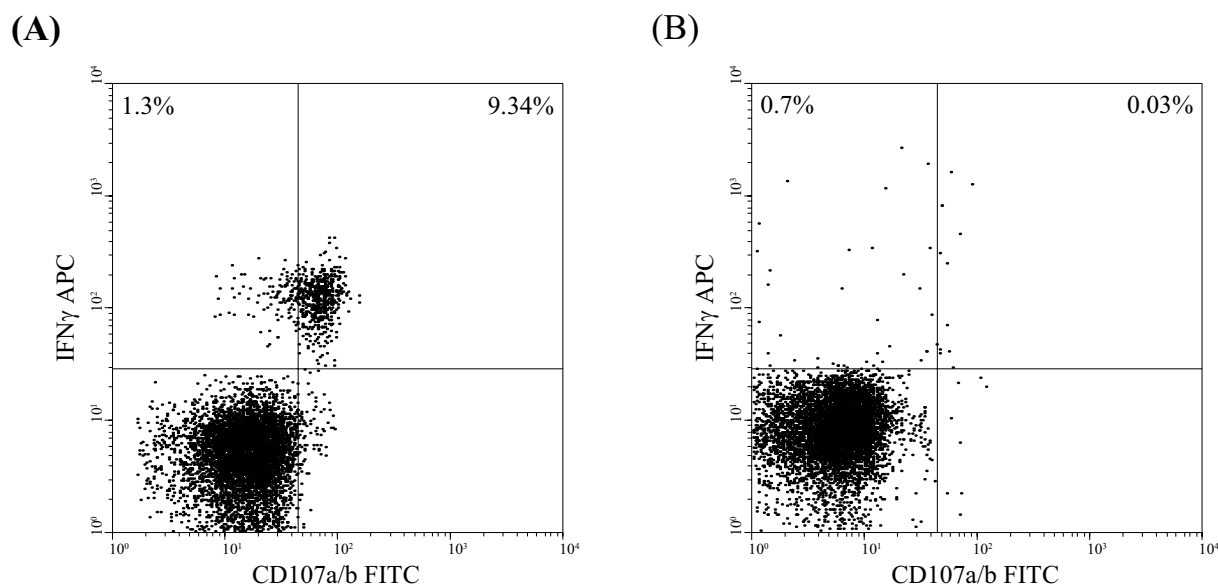


**FIGURE 7.5:** Characterization of antigen-specific CD8<sup>+</sup> T cells. Pooled PBMC from NMRI mice immunized once with (A) 10  $\mu$ g P40-Puu118 or (B) 10  $\mu$ g rP40, were stimulated ex vivo with SEB (*Staphylococcus enterotoxin B*, positive control) or Puu-N peptides and incubated with antibodies to CD107a and b FITC in the presence of Brefeldin A and monensin, then stained with CD3, CD4, and CD8. Events shown are gated for CD3 and CD8.

## 5. DISCUSSION

We cloned and expressed different Puumala virus rNp constructs in the *E. coli* mutant ICONE 200 and evaluated these constructs in outbred NMRI mice as possible vaccine candidates. Several research groups have used yeast-expressed hantavirus rNp constructs together with complete (CFA) and incomplete (IFA) forms of Freund's adjuvant, to evaluate its immunogenicity and use for hantavirus prevention (Dargeviciute et al., 2002; de Carvalho et al., 2002; Klingstrom et al., 2004; Geldmacher et al., 2004). The major disadvantage of CFA

and IFA is the applicability in human vaccination. For this reason rP40, the recombinant outer membrane protein A of *Klebsiella pneumoniae*, was included in our Puumala virus rNp constructs. The rP40 adjuvant, which can possibly be used in human vaccines (Centre d'Immunologie Pierre Fabre, personal communication), induces a mixed IFN $\gamma$ -secreting Th1 cell and IL4-secreting Th2 cell response (Rauly et al., 1999). Our experiments showed that mice vaccinated with the rP40 conjugated rNp constructs, elicited higher antibody titers than mice vaccinated with the Puu-N rNp construct emulsified in Alhydrogel. Moreover, a higher Np-specific antibody response was observed, despite pre-existing anti-rP40 antibodies generated by multiple preimmunizations with the rP40 molecule (data not shown). This observation, together with the fact that no conventional adjuvant is required, makes the use of rP40 vector a particularly practical immunopreventive approach.



**FIGURE 7.6:** Pooled PBMC from NMRI mice immunized once with (A) 10  $\mu$ g P40-Puu118 or (B) 10  $\mu$ g rP40 were stimulated ex vivo with Puu-N peptides in the presence of anti-CD28/CD49d, anti-CD107a and b FITC, and Brefeldin A and monensin. After 6h incubation, cells were stained intracytoplasmatically with IFN $\gamma$  APC. Events shown are gated CD3<sup>+</sup> CD8<sup>+</sup> T cells.

Immunization of NMRI mice resulted in the induction of a strong Np-specific IgG response with a predominance of IgG1 over IgG2b and IgG2a, with high mean IgG1/IgG2a ratios suggesting a predominant Th2 immune response. Furthermore, the mean IgG1/IgG2a ratio was higher for groups of mice treated with rP40-coupled constructs (P40-Puu-N and P40-Puu118 proteins) than for the groups treated with Alhydrogel (Puu-N protein).

All vaccinated mice were intraperitoneally challenged with Puumala virus. As antibodies specific for the nucleocapsid protein do not neutralize virus *in vitro* (Kruger et al., 2001; Maes et al., 2004a), and as there is no lethal Puumala virus animal model available, infection was measured by the presence of neutralizing antibodies, 4 weeks after Puumala virus challenge. In these challenge experiments, we observed complete protection against Puumala virus infection in NMRI mice immunized three times with 10 µg of the P40-Puu118 protein. Surprisingly, 2 µg of the P40-Puu118 protein gave only partial protection after two and three vaccinations, although vaccination with this protein resulted in high antibody titers. The best immunization scheme tested in this study was a three times immunization with 10 µg rNp, which gave the best protection in NMRI mice of 80%, 90% and 100% for respectively Puu-N, P40-Puu-N and P40-Puu118.

It has been shown in literature that antibodies targeting the S segment do not neutralize hantaviruses. In our experiments using the recombinant proteins derived from the S segment, we could confirm this finding. Moreover, hantavirus-neutralizing antibodies are all found to be antibodies derived from the M segment (against the G1 and G2 glycoproteins). In our experiments, we successfully used the absence of neutralizing antibodies to show protection in NMRI mice. Mice protected from Puumala virus challenge by immunization with the recombinant nucleocapsid proteins, did not develop neutralizing antibodies. On the other hand, NMRI mice that were not protected against Puumala virus challenge, had prolonged exposure to the Puumala virus and therefore developed neutralizing antibodies against the G1 and G2 glycoproteins. Our results show that neutralizing antibodies can serve as a good parameter for infection in NMRI mice.

For analysis of cellular responses, we identified antigen-specific CD8<sup>+</sup> T lymphocytes by using flow cytometry. With this technique, CD8<sup>+</sup> T lymphocytes that mediate killing can be identified easily. Using this technique, PUUV-specific T lymphocytes could be detected after a single immunization with 10 µg protein for all three rNp. The P40-Puu118 immunized group showed higher PUUV-specific CTLs over the P40-Puu-N and Puu-N groups which resembles the variations observed in the challenge experiments.

In conclusion, we have shown that immunization with the different Puumala virus rNp constructs resulted in a high and long-lasting Np-specific IgG response. The truncated P40-Puu118 protein gave the best result, with 100% protection after challenge with Puumala virus. Our results indicate that the truncated nucleocapsid protein coupled to rP40, represents a promising candidate for a recombinant subunit vaccine against hantaviruses.

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**CHLOROQUINE, AN ANTI-MALARIA DRUG AS PREVENTION  
FOR HANTAVIRUS INFECTIONS**

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**Piet Maes, Sandra Li, Jan Clement, and Marc Van Ranst.** 2007. Chloroquine, an anti-malaria drug, as prevention for hantavirus infections. Manuscript in preparation.

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## 1. SUMMARY

To date, only ribavirin has been tested as antiviral drug to treat hantavirus infections. Moreover, no WHO-licensed prophylactic vaccine is available yet for prevention. We investigated whether chloroquine, 4-aminoquinoline, a widely used drug with well-known antimalarial effects, could prevent hantavirus infection *in vitro* by using cell culture and *in vivo*, by using the Hantaan virus newborn C57BL/6 mice model. *In vitro* antiviral experiments were performed by using Vero E6 cells and several hantavirus species (Dobrava-Belgrade virus, Hantaan virus, Sin Nombre virus). Hantavirus genomes were detected by using quantitative RT-PCR. For all hantavirus species tested, results indicate that the IC<sub>50</sub> of chloroquine for antiviral activity (mean 10.2 ± 1.43 µM) was significantly lower than its cytostatic activity; CC<sub>50</sub> (mean 260 ± 2.52 µM), yielding an overall selectivity index of 25.5. We also investigated whether chloroquine could prevent death in newborn mice after Hantaan virus infection. Therefore, C57Bl/6 mother mice were treated subcutaneously with daily doses of chloroquine, which is, due to its biochemical characteristics as a lipophilic weak base, excreted in the mother's milk. Subsequently, 1-day-old suckling mice were inoculated intracerebrally with 5 x 10<sup>2</sup> Hantaan virus genomes. In litters of untreated mothers, none of the pups survived Hantaan virus challenge. The highest survival rate (72.7%) of the pups was found when mother mice were treated with a concentration of 10 mg/kg chloroquine. Survival rates declined in a dose dependent manner, with 47.6% survival when treated with 5 mg/kg chloroquine, and 4.2% survival when treated with 1 mg/kg chloroquine.

Our results show that chloroquine can be highly effective against Hantaan virus infection in newborn mice, and may be considered as a future drug against hantaviruses.

## 2. INTRODUCTION

Ribavirin, a broad-spectrum inhibitor of RNA virus replication, is the only causative treatment or prevention available against the primary viral etiology of hemorrhagic fever with renal syndrome (HFRS) or hantavirus pulmonary syndrome (HPS). However, ribavirin is not widely available and should be given only intravenously and early in the clinical course (Chapman et al., 1999; Huggins et al., 1991; McKee Jr. et al., 1988; Severson et al., 2003). This implies that the use of ribavirin intravenously remains limited to quickly recognized and severe forms of disease, where the clinician cannot wait for serological results for his diagnosis and has outweighed the potential benefits of his medication to its non-negligible side-effects. The main treatment of severe HPS or HFRS cases is purely supportive, mostly in

intensive care unit surroundings. This involves mechanical ventilation or even extra-corporeal membrane oxygenation for HPS and all forms of extra-corporeal blood purification (mostly hemodialysis) for HFRS (Clement et al., 2006).

Chloroquine, a 4-aminoquinoline, emerged during the first part of the 20th century as an effective quinine substitute and is still the drug of choice against malaria (Rolain et al., 2007). Chloroquine is a clinically approved drug effective against malaria and is known to elicit antiviral effects *in vitro* against several viruses including hepatitis C virus (Blanchard et al., 2006), HIV type 1 virus (Savarino et al., 2001), influenza virus A and B (Ooi et al., 2006), SARS coronavirus (Vincent et al., 2005; Keyaerts et al., 2004) (Keyaerts et al., 2004) and others (Rolain et al., 2007). Moreover, chloroquine is believed to have immunomodulatory capacities (Jang et al., 2006).

In this study, we evaluated chloroquine for its antiviral potential against the hantavirus species Dobrava-Belgrade virus, Hantaan virus, and Sin Nombre virus *in vitro* and against Hantaan virus *in vivo* in one-day-old C57Bl/6 mice.

### **3. MATERIALS AND METHODS**

#### **3.1 *Virus and cell culture***

The virus strains used in this study were Dobrava-Belgrade virus (DOBV) strain SK/Aa (Klempa et al., 2005), Hantaan virus (HTNV) strain 76-118 (Lee et al., 1978), and Sin Nombre virus (SNV) strain NMR11 (Chizhikov et al., 1995). Vero E6 cells (American Type Culture Collection, C1008) were cultured in minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum. The maintenance medium for virus propagation was identical but contained 2% fetal calf serum. All hantaviruses used in this study were propagated for 10 days on monolayers of Vero E6 cells. Cells and viruses were cultured at 37°C with 5% CO<sub>2</sub>.

#### **3.2 *Compounds***

We tested chloroquine phosphate (7-chloro-4-[[4-(diethylamino)-1-methylbutyl]amino]quinoline phosphate, Alpha pharma, Braine-l'Alleud, Belgium).

### 3.3 Primer and fluorogenic probe design

The S segment nucleocapsid gene of the different hantavirus strains was inspected for primer and probe target sites that would be compatible with Taqman PCR requirements (ABI 7500 Fast Users Manual) by using Primer Express version 2.0 software (Applied Biosystems, Foster City, CA, USA). The primers had matched dissociation temperatures and a minimal likelihood for duplex or hairpin formation. Primer and probe sequences are summarized in TABLE 8.1. The Taqman probes were FAM-TAMRA labeled.

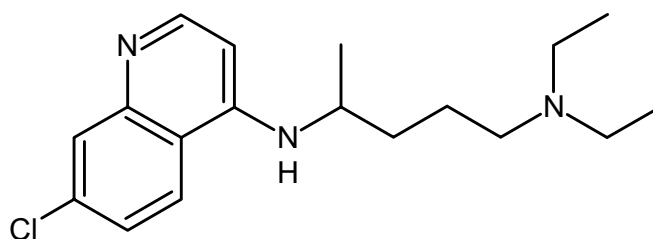


FIGURE 8.1: The structure of chloroquine ( $C_{18}H_{26}ClN_3$ ).

### 3.4 Hantavirus quantitation by using quantitative RT-PCR (qRT-PCR)

qRT-PCR was carried out using the Eurogentec One Step RT qPCR kit (Eurogentec, Seraing, Belgium) with the ABI Prism 7500 Fast Sequence Detection System (Applied Biosystems) as described previously (Maes et al., 2006). Briefly, the reaction was conducted in a 25  $\mu$ l volume containing 5  $\mu$ l of extracted RNA, 12.5  $\mu$ l of One step RT qPCR MasterMix (Eurogentec, Seraing, Belgium) containing ROX as a passive reference, 900 nM forward and reverse primer, 250 nM probe, which was labeled at the 5' end with the fluorescent dye 6-carboxyfluorescein (FAM) as the reporter dye, and at the 3' end with the quencher dye 6-carboxytetramethylrhodamine (TAMRA), and 0.125  $\mu$ l Euroscript/RNase inhibitor (Eurogentec). Reverse transcription was initiated at 48°C for 30 min, followed by PCR activation at 95°C for 10 min and 45 cycles of a two-step incubation at 95°C for 15 s (denaturation) and 60°C for 1 min (primer annealing and elongation). The reporter dye (FAM) signal was measured against the internal reference dye (ROX) to normalize for non-PCR-related fluorescence emissions. The threshold cycle ( $C_T$ ) was defined as the fractional cycle number at which the reporter fluorescence, generated by cleavage of the probe, reaches a threshold defined as 10 times the standard deviation of the mean baseline emission. In order to allow absolute hantavirus quantitation, cRNA standards were constructed and used for the generation of a standard curve, as described previously (Chapter 5).

**TABLE 8.1:** Primer and Taqman probe sequences.

	Forward primer (5' to 3')	Reverse primer (5' to 3')	Taqman probe (5' to 3')
<b>DOBV</b>	TCCCGTGCAAGCTACTATCTGA	GCGCTCCTTGTCTTTGATTCA	ACCAAAGGCCCATCCACCAATCGT
<b>HTNV</b>	CTGGATTAAACCATTGGATATTGA	TATCGGGACGACAAAGGATGTA	AGCAGACTGGCTGAGCATCATCGTCTATCT
<b>SNV</b>	TACTCCTCATTCAAGTTGGGTCTTT	TCCCGGCCACATA TAATGCT	CATGTGCTCCAGATCGTTGTCCACCT

### 3.5 Antiviral assay

Antiviral measurements were based on the reduction of hantavirus titer on Vero E6 cells. Monolayers of Vero E6 cells were infected with  $10^4$  hantavirus copies per ml in the presence of various concentrations of chloroquine (ranging from 0.032 to 500  $\mu$ M). After 5 days of incubation at 37°C 5% CO<sub>2</sub>, cell supernatants were collected and the viral titer was determined by using the qRT-PCR described above.

### 3.6 Cytotoxicity assay

Cytotoxicity measurements were based on the viability of Vero E6 cells in the presence of various concentrations of chloroquine as previously described (Keyaerts et al., 2004). After a five days incubation period, the number of viable cells was quantified by a tetrazolium-based colorimetric method, in which the reduction of MTS (CellTiter 96 AQueous One Solution kit, Promega, The Netherlands) by mitochondrial dehydrogenases to a soluble colored formazan was measured in a spectrophotometer at 492 nm. The cytotoxic concentration was defined as the concentration of the compound that reduced cell viability by 50% (50% cytotoxic concentration, CC<sub>50</sub>).

### 3.7 In vivo evaluation of chloroquine in 1-day-old C57Bl/6 pups

Six-week-old male and female C57Bl/6 mice were obtained from Elevage Janvier (Elevage Janvier, Le Genest Saint Isle, France). Pregnant mice were treated daily with several dilutions of chloroquine subcutaneously, starting with the treatment 2 days before birth of the pups. Within 24h of birth, neonatal C57Bl/6 mice were inoculated intracerebrally with  $5 \times 10^2$  Hantaan virus particles. The surviving mice were counted for up to 60 days after the infection. All mice were treated according to the laboratory animal control guidelines of our institute,

which conform to those of the European Commission. All animal experiments were carried out in a BSL-3 facility.

#### 4. RESULTS AND DISCUSSION

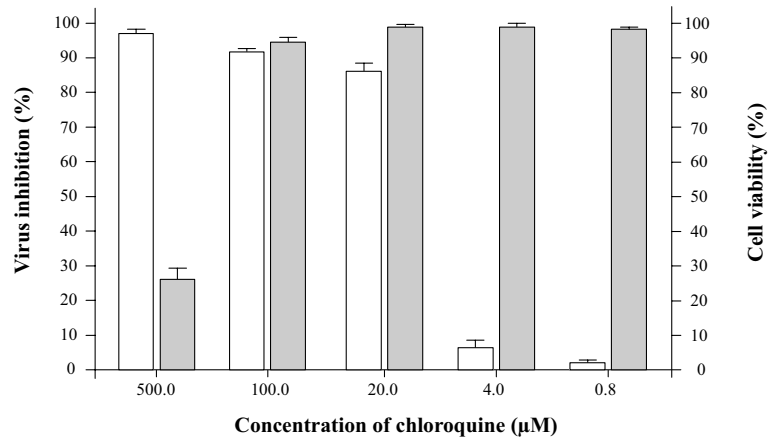
In this study we report the *in vitro* and *in vivo* antiviral activity of chloroquine against hantavirus infections. The *in vitro* activity of chloroquine was tested on Vero E6 cells. Cytotoxicity in these cells was measured in parallel with the antiviral activity. These experiments, done in triplicate, were repeated three times. In the virus yield assays, where viral RNA was quantified using qRT-PCR 7 days postinfection, the IC<sub>50</sub> was 9.51 μM, 10.49 μM and 10.47 μM for respectively Dobrava virus, Hantaan virus, and Sin Nombre virus (TABLE 8.2).

**TABLE 8.2:** Overview of the antiviral activity of chloroquine against Dobrava virus, Hantaan virus, and Sin Nombre virus with their 50% inhibitory concentration (IC<sub>50</sub>), 50% cytotoxic concentration (CC<sub>50</sub>), and selectivity index (SI)

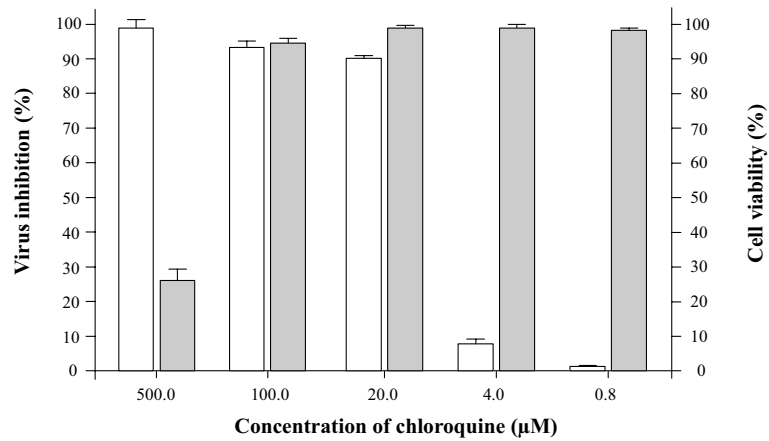
	IC <sub>50</sub> (μM)	CC <sub>50</sub> (μM)	SI
<b>Dobrava virus</b>	9.51 ± 1.1	260 ± 2.52	27.3
<b>Hantaan virus</b>	10.49 ± 1.8	260 ± 2.52	24.8
<b>Sin Nombre virus</b>	10.47 ± 1.4	260 ± 2.52	24.8

We also investigated whether chloroquine could prevent death in newborn C57Bl/6 mice as a result of infection with Hantaan virus. Hantaan virus and Seoul virus infections of suckling C57Bl/6 mice have been reported to be lethal, although without reflecting the symptoms seen in humans (Yoo et al., 1993). The primary target of infection is the same in newborn C57Bl/6 mice as in humans, namely the capillary endothelium (Kurata et al., 1983). In the *in vivo* experiments, C57Bl/6 mother mice were treated subcutaneously with daily doses of chloroquine, which is, due to its biochemical characteristics as a lipophilic weak base, excreted in the mother's milk. Treatment was started in these mother mice 2 days before birth and stopped 16 days later. The mice were followed for 60 days. Subsequently, 1-day-old suckling mice were inoculated intracerebrally with  $5 \times 10^2$  Hantaan virus particles. In litters of

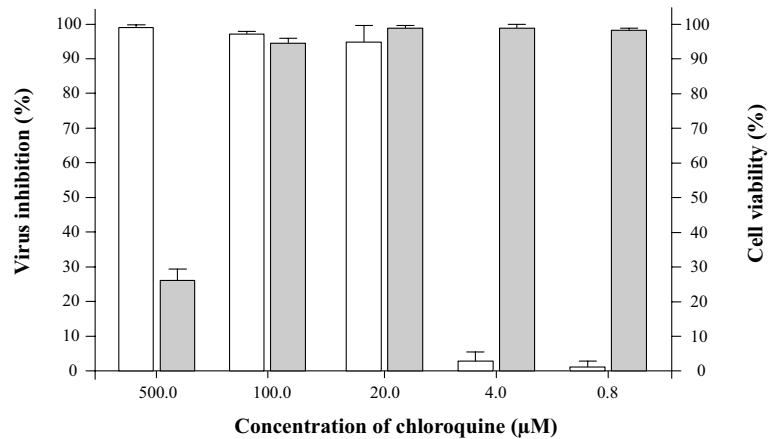
**(A) Dobrava virus**



**(B) Hantaan virus**

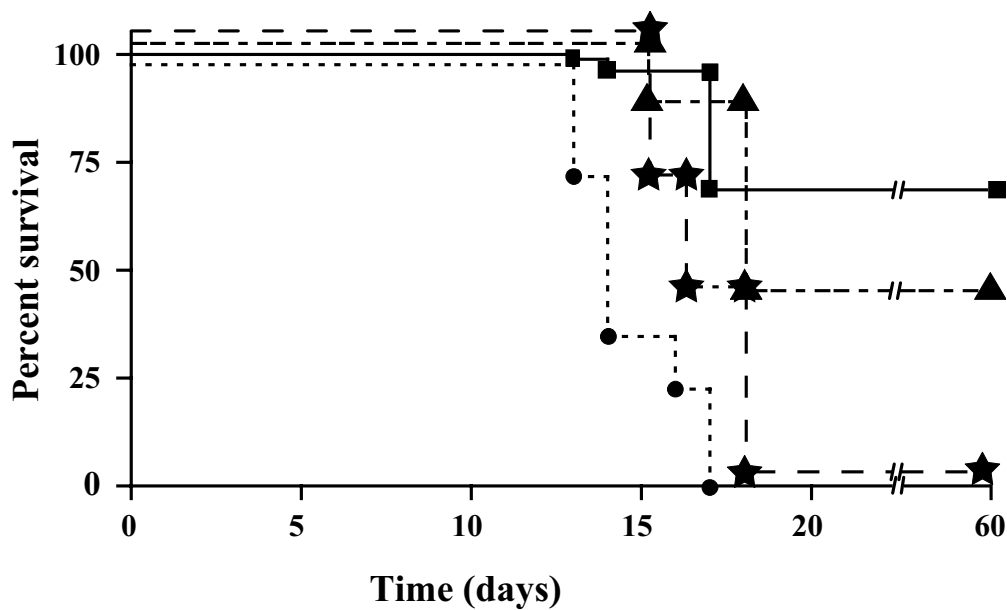


**(C) Sin Nombre virus**



**FIGURE 8.2:** Hantavirus-infected Vero E6 cells were incubated for 5 days in the presence of different concentrations of chloroquine. White bars indicate the effect of chloroquine on the hantavirus replication (left Y-axis). Black bars show the effect of chloroquine on viability of mock infected cells (right Y-axis). The concentration of chloroquine that results in 50% inhibition of the virus,  $IC_{50}$ , was 9.51  $\mu\text{M}$ , 10.49  $\mu\text{M}$  and 10.47  $\mu\text{M}$  for respectively (A) Dobrava virus, (B) Hantaan virus and (C) Sin Nombre virus.

untreated mothers, none of the pups survived virus challenge. The highest survival rate (16 out of 22 pups, 72.7%) of the pups was found when mother mice were treated with daily doses of 10 mg/kg chloroquine. Survival rates declined in a dose dependent manner, with 47.6% survival when treated with 5 mg/kg chloroquine (10 out of 21 pups), and 4.2% survival (1 out of 24 pups) when treated with 1 mg/kg chloroquine (FIGURE 8.3). Daily doses of chloroquine greater than 10 mg/kg resulted in death of the offspring.

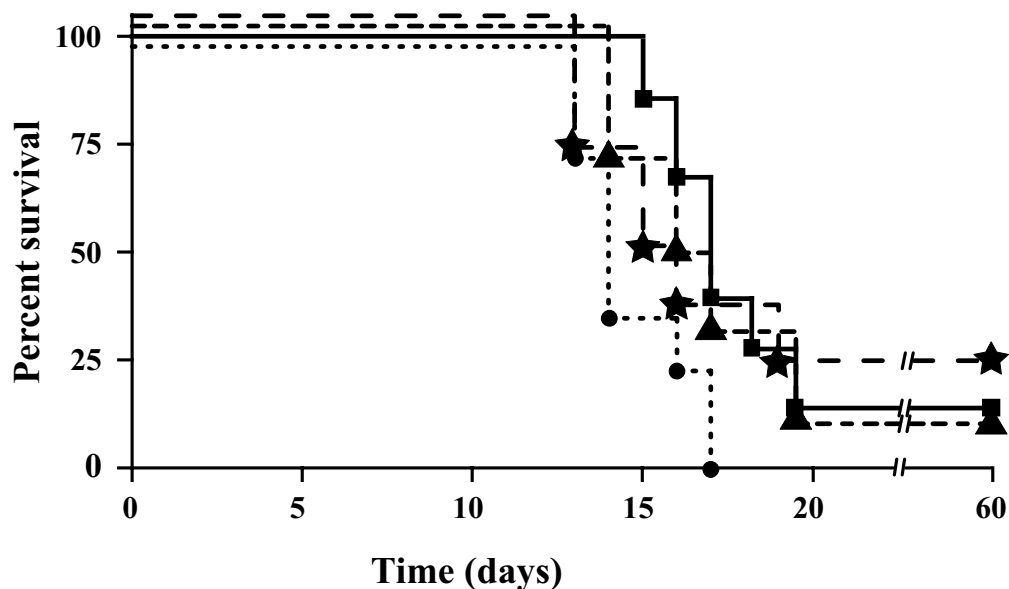


**FIGURE 8.3:** The preventive effect of chloroquine on Hantaan virus infection in newborn mice. Mothers were treated daily with different concentrations of chloroquine for 16 days. Treatment started 2 days before birth. (●) PBS; (★) 1 mg/kg chloroquine, (▲) 5 mg/kg chloroquine, (■) 10 mg/kg chloroquine.

Moreover, if treatment with 10 mg/kg chloroquine of C57Bl/6 mother mice was started after infection, the litters showed 16.7% survival (3 out of 18 pups, FIGURE 8.4). Furthermore, if mothers were treated only before birth of the pups (treatment with chloroquine was stopped before infection), survival of the pups was 12.5% (2 out of 16 pups, FIGURE 8.4). Daily treatment of 1-day-old suckling mice from untreated mothers with chloroquine subcutaneously, induced only partial protection (3 out of 12 pups survived, 25%). Doses of chloroquine higher than 7 mg/kg given subcutaneously to suckling C57Bl/6 mice were found to be lethal.

Our results indicate that chloroquine is effective as prophylaxis against hantavirus infections, however, we have not enough substantiation that chloroquine can be used as treatment for these infections. This in contrast with ribavirin, which is effective against Hantaan virus in

C57Bl/6 pups, when treatment is started as late as day 10 after infection (Huggins et al., 1986). Hantaviruses are spread to humans via aerosolized excreta from chronically infected wild rodents. Puumala virus can survive and can be transmitted to other rodents for up to 15 days after being excreted (Kallio et al., 2006) whereas Hantaan virus (HTNV) can remain infectious under optimal conditions for up to 96 days (Hardestam et al., 2007). *Myodes glareolus* or bank vole, the rodent host of Puumala virus, prefers to make its burrows under stack of wood or near the forest (Van Loock et al., 1999). Therefore, chloroquine, which is intensively studied and has limited and well-known side effects, can be of great importance as prophylactic medication for people living in and traveling to hantavirus affected areas. This medicine is ubiquitously available, of low cost, and easy to administer. It may be considered for immediate use in the prevention and treatment of hantavirus infections.



**FIGURE 8.4:** The therapeutic effect of chloroquine on Hantaan virus infection in newborn mice. Symbols: (●) PBS; (■) mothers treated with daily doses of 10 mg/kg chloroquine started after infection (16.7% survival); (▲) mothers treated with 10 mg/kg chloroquine before birth of the pups (day -2 and -1, 12.5% survival); (★) pups treated daily with 10 mg/kg chloroquine subcutaneously (25% survival).

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**GENERAL DISCUSSION AND REFLECTION**

### 1. HANTAVIRUS NUCLEOCAPSID PROTEIN-BASED VACCINE

The high number of HFRS cases, especially in Asia, indicates an urgent need to develop a safe, effective vaccine against hantaviruses. This urgency has prompted researchers to produce vaccines by the same traditional inactivated-virus approaches that have been proven efficacious for other viral pathogens. There is one commercially produced vaccine, Hantavax, available in South Korea. This vaccine was developed by serial passage of Hantaan virus in brains of suckling mouse, followed by inactivation with formalin. Initial claims reported that two doses of the vaccine, given via a subcutaneous route one month apart, resulted in 100% seroconversion as measured by immunofluorescence (Cho et al., 2002; Hjelle, 2002). The production of this vaccine, however, was not in compliance with current U.S. Food and Drug Administration guidelines on "Good Manufacturing Practices" and the protective efficacy has not yet been determined. Furthermore, adventitious agents in the product were not rigorously excluded and the animal colony used for the vaccine's development was not pathogen-free.

The human IgG immune response in HFRS patients is primarily directed against epitopes located within the amino acid residues 1 to 119 of the amino-terminal part of the nucleocapsid protein (Gott et al., 1997). Moreover, cytotoxic T lymphocyte epitopes of PUUV and other hantaviruses are mainly located in the same region of the nucleocapsid protein (de Carvalho et al., 2001; Ennis et al., 1997; Van Epps et al., 1999). Bulk T lymphocyte responses are directed against the Hantaan virus nucleocapsid protein. One study recognized Hantaan virus-specific CD8<sup>+</sup> epitopes to the nucleocapsid protein and CD4<sup>+</sup> epitopes to G1 protein (Van Epps et al., 1999). In mice challenged with PUUV, several T helper lymphocyte recognition sites were identified, spanning amino acids 6-27, 96-117, 211-232, and 256-277 of the PUUV nucleocapsid protein (de Carvalho et al., 2001). All these regions have been shown to react with antibodies in human NE sera (Vapalahti et al., 1995).

The mechanism by which vaccination with exogenously expressed nucleocapsid proteins protects against infection remains unknown. Although vaccination with the nucleocapsid protein elicits antibody responses, in most cases these antibodies fail to efficiently neutralize virus *in vitro*. One report showed that vaccination with certain fragments of *E. coli*-expressed PUUV nucleocapsid proteins elicited low-titer neutralizing antibody in bank voles, as measured by focus reduction assay (Lundkvist et al., 1996). Protection in the absence of high-titer anti-nucleocapsid neutralizing antibodies suggests that a protective cell-mediated (i.e.

cytotoxic T lymphocytes) immune response could be elicited by vaccination with exogenously expressed nucleocapsid proteins. In our experiments, recombinant proteins derived from the nucleocapsid protein of DOBV and PUUV showed to be highly immunogenic, especially the proteins consisting of the first 118 amino-terminal amino acids and associated to the rP40 molecule. Moreover, the potential of these recombinant proteins to generate a cytotoxic T lymphocyte response could be shown. These antigen-specific T lymphocytes were enumerated by using a flow cytometric detection assay, which measures the exposure of CD107a and b, present in the membrane of cytotoxic granules, onto the cell surface as a result of degranulation. This technique has the advantage over other methods, such as ELISpot analysis, in that it allows for precise phenotypic characterization of the T lymphocyte populations of interest. CD8<sup>+</sup> T cell-mediated target cell killing, however, has historically been assessed by the standard chromium (<sup>51</sup>Cr) release assay (Brunner et al., 1968), or, more recently, by methods that monitor the release of fluorescent dyes from target cells (Sheehy et al., 2001; Liu et al., 2002). These techniques are cumbersome, semi-quantitative and potentially insensitive. Importantly, none of these methods directly examine the CD8<sup>+</sup> T cells that mediate killing; rather, they examine the death of target cells, essentially the aftermath of CD8<sup>+</sup> T cell effector function.

## **2. ANTIVIRAL THERAPY FOR HANTAVIRUS INFECTIONS**

The antiviral drug ribavirin, when given in early treatment, has proven beneficial for the therapy of severe Far Eastern cases (Huggins et al., 1991). Intravenous injection of ribavirin in the early stages of the disease was shown to reduce mortality and the severity of symptoms. Otherwise, management of all HFRS patients is highly individualized and focused on supportive care (Clement et al., 1997). Moreover, the drug seems less suited for the mostly mild PUUV-induced cases (Clement, 2003). Management of HPS includes hospitalization and intensive care, fluid therapy, administration of vasopressors and antibiotics, and close monitoring of oxygenation. Ribavirin has failed to show efficacy in HPS (Kruger et al., 2001; Hjelle et al., 1995; Peters et al., 1999). Chloroquine is a 9-aminoquinoline known since 1934, which emerged during the first part of the 20th century as an effective quinine substitute and the drug of choice against malaria (Wellems and Plowe, 2001). Concomitant with a gradual decrease in its use for therapy and prophylaxis of *Plasmodium*-induced disease worldwide, related to the emergence of chloroquine-resistant parasites, chloroquine and its hydroxyl

analogue (hydroxyl chloroquine) have gained interest in the field of other infectious diseases (Savarino et al., 2001; Savarino et al., 2004). Chloroquine is a weak base that increases the pH of acidic vesicles. When added extracellularly the non-protonated portion of chloroquine enters the cell where it becomes protonated and concentrated in acidic, low-pH organelles, such as endosomes, Golgi vesicles, and lysosomes (Savarino et al., 2006). Chloroquine can affect virus infections in many ways and the antiviral effect depends in part on the extent to which the virus utilizes endosomes for entry. Although we have shown a positive effect of chloroquine on the outcome of Hantaan virus infected newborn C57Bl/6 pups (Chapter 8), the mechanism of action remains unclear. Basic questions concerning hantavirus maturation remain open. We are only beginning to understand some of the cellular mechanisms by which the hantaviruses mature into infectious particles. It is still not clear if hantavirus maturation occurs intracellularly or at the cell surface. The majority of the negative stranded RNA viruses have been shown to assemble and bud from the cell surface (Cadd et al., 1997; Spiropoulou, 2001). In contrast, viruses of the family Bunyaviridae are characterized by an unusual pattern of intracellular maturation where the bilipid envelope of the virus particles is derived from the membranes of the endoplasmic reticulum-Golgi compartment (Murphy et al., 1973; Spiropoulou, 2001). Due to this characteristic feature, Golgi maturation has been used as one of the criteria for classification of viruses as members of the family Bunyaviridae. Intracellular maturation of hantaviruses can be observed, but not as easily as with some other members of the family Bunyaviridae. EM studies on Sin Nombre virus and Black Creek Canal virus (both New World hantaviruses) however, have suggested that the maturation of these viruses did not occur intracellularly in the Golgi compartment. This conclusion was based on the observation that the majority of the virus particles were present extracellularly, with only rare virus-like particles observed inside the cell (Spiropoulou, 2001). Yet, EM pathogenesis studies have revealed intracellular hantavirus-like particles in the microvascular lung endothelium and interstitial macrophages of HPS patients (Zaki et al., 1995). Moreover, in trafficking studies it has been shown that, in both New and Old World hantaviruses, the expressed nucleocapsid and glycoproteins are accumulating intracellularly in the Golgi apparatus and that they could not be detected at the cell surface (Betenbaugh et al., 1995). Alternatively, in advanced stages of virus infection, there is undeniable detection by immunofluorescence staining of substantial amounts of glycoproteins at the cell surface (Spiropoulou, 2001). Although these findings may suggest a different maturation for New

World and Old World hantaviruses, it is clear that more studies are needed before definitive conclusions can be drawn.

In our experiments, chloroquine was tested *in vitro* on Vero E6 cells with the Old World hantaviruses Dobrava-Belgrade virus and Hantaan virus, and the New World hantavirus Sin Nombre virus. For all three viruses the 50% inhibitory concentration (IC<sub>50</sub>) was significantly lower than the 50% cytotoxic concentration (CC<sub>50</sub>), with an overall selectivity index of 25.5. No difference in reactivity of chloroquine could be seen between the New World virus Sin Nombre virus and the Old World viruses Dobrava-Belgrade virus and Hantaan virus. Moreover, with the infection experiments of newborn C57Bl/6 mice with Hantaan virus, it could be concluded that chloroquine can be used in the prevention of infection rather than in the treatment of infection. This discrepancy however, can be due to the use of the specific animal model. Although the primary target of Hantaan virus, the capillary endothelium, is the same in newborn C57Bl/6 mice as in humans, this animal model represent an infection model and not a disease model since it does not reflect the symptoms seen in humans. A practical animal model for HPS, would be the Syrian hamster model. Andes virus is very lethal in adult Syrian hamsters. The characteristics of the disease in these hamsters, including the incubation period, symptoms of rapidly progressing respiratory distress and pathologic findings of pulmonary edema and pleural effusion, closely resemble HPS in humans (Hooper et al., 2001; Wahl-Jensen et al., 2007). Unfortunately, due to the high mortality rates following Andes virus infections, with persuasive evidence of person-to-person transmission (Wells et al., 1997), these experiments would have to be performed in a biosafety level 4 facility to which only very few laboratories have access.

One of the major advantages of chloroquine is its limited and preventable toxicity. Long experience of the use of this drug in the treatment of malaria has already demonstrated the safety of short-term administration to humans. In theory, chloroquine can be used immediately for the prevention of hantavirus infection. It has been already widely used for chronic administration in rheumatic diseases and for antimalarial prophylaxis for up to several years with only a low incidence of adverse effects, even during pregnancy (Rolain et al., 2007).

### 3. CLASSIFICATION OF HANTAVIRUSES

Hantavirus diseases are present in most of Europe, Asia and the Americas, and diagnosing them depends on clinical alert and use of serological tests. By mapping endemic areas with more detailed surveillance of rodents, development of more rapid and sensitive tests, and increased clinician awareness, human hantavirus infections will presumably be detected in new areas and new rodent species might be seen to carry yet unknown hantaviruses. The status and future of hantavirus vaccines and antiviral therapy varies greatly among different countries, which generally need to be considered individually. The nature of the endemic and epidemic threats, the tolerance of the population and the regulatory agencies for vaccines of differing quality and the socioeconomic status of the population are but some of the factors that play this equation (Hjelle, 2002). Hantavirus infections are clearly preventable at least at some level by vaccines, but no vaccine has gained widespread acceptance. Thus far, no vaccine has been proven to be capable of eliciting protection for a protracted period, while maintaining an acceptably low side-effect profile and with a reasonably convenient regime of immunization and booster immunization. Since the hantaviruses are fairly antigenically diverse, it is likely that at least two and possibly three or more vaccine components would need to be prepared to protect against all members of the genus (Hjelle, 2002). However, the only 'solid' characteristics of many hantavirus species still exist in the form of nucleotide sequences of their genome, partial sequences in most cases. Moreover, the rapid progress during the last two decades in gene amplification and sequencing techniques has made genetic approaches even more appealing to virologists who nowadays can perform initial genetic characterization of new virus variants, strains and species in a relatively short period of time. The lack of appropriate rules for genetic classification of the species, however, will unavoidably lead to incorrect identification of virus species and to wrong assessments of the geographical spread of hantavirus species. In Chapter 2, we discussed the applicability of similarity analyses in the determination of the hantavirus species. Our results demonstrate that by using nucleotide sequences, no clear definition of hantavirus species can be put forth. Moreover, nucleotide differences do not always correlate with amino acid differences, which make it highly questionable whether nucleotide sequences can result in relevant classification of the virus. We showed that hantavirus species can be distinguished by an amino acid distance greater than 10.0% (Poisson correction model). One could argue that this simplified genetic approach for demarcating hantavirus species does not take into account the 'biological' properties, i.e. their reservoir and their reaction pattern in neutralization tests. It

should be clear that for a full and academic understanding both characteristics remain necessary, hence the requirement of a confirmed rodent (or insectivore) host and of a proper virus isolate. The question arises, however, if for a quick and readily accessible classification technique, these data are an absolute prerequisite. The paradigm “one rodent species for one hantavirus species” has been breached and, for diagnostic purposes, neutralization tests are rarely required to make a distinction between closely related hantavirus species (if at all possible). Furthermore, we have to keep in mind that the first and sometimes subsequent findings of a particular hantavirus in a particular rodent host may not indicate that this represents the principal virus cycle in nature; in such situations additional studies are necessary to determine the primary and secondary or accessory cycles. Additionally, mammalian taxonomy is as unsettled a field of study as is viral taxonomy, the strict adherence to a requirement for a unique rodent host for each viral species is no more reasonable than a strict requirement for a specific gene sequence similarity.

#### **4. CONCLUDING REMARKS**

Hantaviruses have presumptively caused disease for centuries before yielding clues to their epidemiology and ecology over the last few decades. Although these viruses are ‘old’, environmental changes may affect the geographic distribution and dynamics of the carrier rodent species and hence the epidemiology of hantaviruses. Continuing scientific studies will help to dissect the long-term sequelae, pathogenesis, transmission and distribution of these viruses and their carriers, and hopefully lead to more effective treatments and prevention strategies.



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