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Nucleocapsid protein of cell culture-adapted Seoul virus strain 80-39: Analysis of its encoding sequence, expression in yeast and immuno-reactivity

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Abstract. Seoul virus (SEOV) is a hantavirus causing a mild to moderate form of hemorrhagic fever with renal syndrome that is distributed mainly in Asia. The nucleocapsid (N) protein-encoding sequence of SEOV (strain 80-39) was RT-PCR-amplified and cloned into a yeast expression vector containing a galactose-inducible promoter. A survey of the pattern of synonymous codon preferences for a total of 22 N protein-encoding hantavirus genes including 13 of SEOV strains revealed that there is minor variation in codon usage by the same gene in different viral genomes. Introduction of the expression plasmid into yeast *Saccharomyces cerevisiae* resulted in the high-level expression of a hexahistidine-tagged N protein derivative. The nickel-chelation chromatography purified, yeast-expressed SEOV N protein reacted in the immunoblot with a SEOV-specific monoclonal antibody and certain HTNV- and PUUV-cross-reactive monoclonal antibodies. The immunization of a rabbit with the recombinant N protein resulted in the induction of a high-titered antibody response. In ELISA studies, the N protein was able to detect antibodies in sera of experimentally infected laboratory rats and in human anti-hantavirus-positive sera or serum pools of patients from different geographical origin. The yeast-expressed SEOV N protein represents a promising antigen for development of diagnostic tools in serology, sero prevalence studies and vaccine development.

Key words: codon usage, ELISA, hantavirus, monoclonal antibodies, nucleocapsid protein, Seoul virus, yeast expression

Introduction

Hantaviruses represent a separate genus *Hantavirus* in the family *Bunyaviridae*. They can cause two types of diseases in humans, "Hantavirus Cardiopulmonary Syndrome" (HCPS) in the Americas and "Hemorrhagic Fever with Renal Syndrome" (HFRS) mainly on the Eurasian continent [for reviews see [1,2]]. The prototype virus of the genus *Hantavirus*, the Hantaan virus (HTNV), carried by the striped field mouse (*Apodemus agrarius*), was identified as the causative agent of Korean Hemorrhagic Fever (KHF), a severe form

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of HFRS, with a case fatality ratio of up to 10% due to severe bleeding, shock and renal failure [3,4]. Milder clinical courses of usually urban HFRS with a case fatality ratio of about 1% are caused by infections with the closely related Seoul virus (SEOV) carried by different species of the genus *Rattus*, i.e. *R. rattus*, *R. norvegicus* and *R. losea* [4–7].

In general, hantaviruses are transmitted from persistently infected rodent reservoir hosts to humans by inhalation of virus-contaminated aerosols originating from rodent excreta. In fact, data from experimental infection of urban rats (*R. norvegicus*) and investigations in an enzootic focus of SEOV-infected rats suggested the important role of virus-contaminated urine in the transmission of SEOV [8]. An additional transmission mode in male rats may be associated with aggression and wounding that is caused by increased testosterone levels [9].

SEOV infections have been reported from different Asian countries, i.e. Korea, China, Japan, (Far East) Russia, Indonesia and Cambodia [6,10–15]. However, SEOV is the only "cosmopolitan" hantavirus known so far and represents an example of global anthropogenic expansion of an infectious agent [16]. This is supported by the detection of human SEOV infections in other countries outside Asia, e.g. in the USA, Baltimore area [17] and Brazil [18], and circulation of SEOV in rat populations from Australia, USA, Germany, Northern Ireland, Indonesia and Greece [19–24]. Furthermore, infections by rat-borne hantaviruses have been reported in medical research institutions where laboratory rat colonies were handled [25]. In contrast, recent studies in Central Europe failed to detect human SEOV infections [26].

In general, diagnostics of human hantavirus infections is based on serological methods, i.e. immunofluorescence, ELISA and immunoblot tests. As an alternative to serological tests based on virus antigen or virus-infected cells, highly specific and sensitive tests were developed on the basis of heterologously expressed nucleocapsid (N) proteins. To prevent specificity problems associated with *E. coli*-expressed recombinant N (rN) protein [27], we have recently generated rN proteins of different hantaviruses, namely HTNV, different strains of Puumala virus (PUUV), Sin nombre virus (SNV), Andes virus (ANDV) and Dobrava virus strains associated to *Apodemus agrarius* (strain Slovakia; DOBV-Slk) and *A. flavicollis* (strain Slovenia; DOBV-Slo) in yeast *Saccharomyces cerevisiae* for use in diagnostic assays [28,29, J. Schmidt et al., submitted for publication].

The objective of the present study was the yeast expression and characterization of SEOV strain 80-39 rN protein for a potential use in diagnostic assays and vaccine applications. To prove if this rN protein can be taken in serological investigations as a representative of the SEOV species, the N protein-encoding sequence of Korean strain derivative 80-39-Berlin (80-39-B), cell culture-passaged in the biosafety level 3 laboratory in Berlin, was determined and its phylogenetic relationship to other SEOV strains was analyzed, most importantly to exclude a potential influence of nucleotide and resulting amino acid sequence alterations due to cell culture passaging. The purified SEOV rN protein was used to study its immuno-reactivity using monoclonal antibodies, sera of experimentally SEOV-infected rats, rabbits immunized with hantavirus rN proteins and human sera or serum pools from HFRS and HCPS patients.

Materials and Methods

Rat Sera und Tissue Samples

The generation of serial follow-up serum samples from four experimentally SEOV (strain SR-11)-infected Wistar rats has been described previously [30]. As negative control, sera from five wild-trapped rats from Japan, previously demonstrated to be non-infected [31], were used. In addition sera from 11 brown rats (*R. norvegicus*) trapped in Westphalia, Germany, were included in the study.

Total DNA from liver tissue samples of two wild-trapped brown rats from Germany was used for mitochondrial (mt) DNA characterization.

DNA Isolation, PCR Amplification and Determination of Rat mt DNA Sequences

Total rat DNA was isolated using the QIAamp DNA Mini Kit according to the protocol of the manufacturer (QIAGEN, Hilden, Germany). The 12S rDNA sequence was PCR-amplified using the

primers L1091 (5′-GGGATTAGATACCCCAC-TAT-3′) and H1478 (5′-TGACTGCAGAGGGT-GACGGGCGGTGTGT-3′) from the isolated rat DNA and sequenced using the ABI Prism Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol on ABI 377 DNA sequencers.

RNA Isolation, RT-PCR, Cloning and Sequencing of the SEOV N Protein-encoding Sequence

Vero E6 cells were infected with SEOV (strain 80-39) [5]. Infected cells as well as cell culture supernatant were treated with Trizol reagent (Gibco BRL, Eggenstein, Germany). The suspensions were used to isolate viral RNA with the RNeasy Kit (QIAGEN), according to the recommendations of the manufacturer. After reverse transcription of the RNA by RevertAid H Minus M-MuLV Reverse Transcriptase (MBI Fermentas, St. Leon-Rot, Germany) with the Seoul-S-RT Primer 5′-TAGTAGTAGACTCCCT AAA-GAGCTA-3′, the N protein-encoding sequence (aa 2-429) of the cDNA copy was PCR-amplified using Pfu DNA Polymerase (Stratagene, Amsterdam, The Netherlands) with the SEOV-N Forward Primer 5′-AAGCTAGCGCAACTATG GAAGAAATCCAGAGAG-3′ and the SEOV-N Reverse Primer 5′-AAGCTAGCTTATAATTTC ATAGGTTCCTGGTTTGA-3′, containing unique sites for *Nhe*I. The PCR amplification products were purified and subsequently inserted into the plasmid pSTBLUE-1 with the Perfectly Blunt Cloning Kit (Novagen, Schwalbach, Germany), according to the recommendations of the manufacturer. Plasmids were purified using the Spin Mini Kit (QIAGEN). DNA sequencing of three plasmids was performed using an ABI Prism Big Dye terminator cycle sequencing kit (Applied Biosystems). The N protein-encoding sequences obtained for three pSTBLUE-1/SEOV-N plasmids were identical.

Codon Use Analyses

The DnaSP 4.0 program [32] was used to calculate codon usage tables as well as the G + C content at the second (G + C2), third (G + C3), and all coding positions (G + Cc) for each N protein-encoding sequence analysed. The same program was also used to calculate different codon bias measures: the Relative Synonymous Codon Usage (RSCU; [33]) values, the Effective Number of Codons (ENC; [34]), the Codon Bias Index (CBI; [35], and the Scaled Chi square (SChi2; [36]) values. To examine the similarities of a set of genes in terms of their similarity of codon usage, we followed McInerney's approach implemented in the GCUA program [37]. A distance matrix was produced from all the N protein-encoding sequences based on the average differences in their RSCU values. The analysis was continued with a tree-like representation of this RSCU distance matrix produced using PAUP* and comparing that to the ML phylogeny of the sequences based on a substitution model.

Phylogenetic Analysis of Nucleotide and Amino acid Sequences

For phylogenetic analysis, the sequences were aligned on amino acid level and then reverse-translated to nucleotide sequences using DAMBE software [38]. DotPlot analysis implemented in BioEdit software package [39] was used to check the reliability of the alignment. We carried out two types of phylogenetic analysis to investigate evolutionary relationships using PAUP* 4.0b10 [40]: (i) neighbour-joining (NJ) based on LogDet/paralinear distances which were designed to deal with unequal base frequencies in each pairwise sequence comparison – thus it allows base compositions to vary over the tree [41] and (ii) maximum-likelihood (ML) analysis. Before the ML analyses, we used likelihood ratio tests and the computer application MrModeltest v1.0b. [42] to determine the best-suited model of sequence evolution. The best-fit model selected by MrModeltest v1.0b. for the N protein-encoding sequence data set and then used to reconstruct phylogenetic trees was the general time-reversible model [43] with an allowance for invariant sites and a gamma shape for among-site rate variation under the hierarchical likelihood ratio test method. Heuristic ML searches were performed with 10 replicates of random sequence addition and TBR branch swapping. Non-parametric bootstrap analyses with 100 pseudo-replicates were performed to obtain estimates of support for each node of the ML tree; NJ bootstraps employed 1000 iterations.

Generation of the Yeast Expression Plasmid

The SEOV-N-encoding sequence was isolated from pSTBLUE-1/SEOV-N as an *Nhe*I fragment and inserted into *Xba*I-linearized, CIP-dephosphorylated plasmid pFX7-His. This plasmid represents a derivative of pFX7 that contains a Gal/Pyk hybrid promoter, a formaldehyde resistance gene and in front of the unique *Xba*I insertion site an MHHHHHH-coding sequence [28,29]. The DNA sequence in the joining region of pFX7-His plasmid and the SEOV N-encoding insert in the expression plasmid pFX7-His-SEOV-N was confirmed by DNA sequencing using primer pyk5 (5′-TTCTTTTTCATCCTTTGG-3′).

Expression and Purification of SEOV rN protein

The recombinant plasmid pFX7-His-SEOV-N was introduced into competent yeast *S. cerevisiae* cells of the haploid strain AH22 derivate 214 (*ura3 leu2 his4*). Expression and purification of the His-tagged rN protein was performed according to a protocol described recently for rN proteins of other hantaviruses [28,29]. Briefly, yeast cells were grown in formaldehyde-containing YEPD medium. Synthesis of the SEOV rN protein was induced by adding galactose. Yeast cells were pelleted by centrifugation and disrupted using glass beads. The His-tagged SEOV rN protein, enriched by centrifugation steps, was finally purified using nickel-chelate resin according to the protocol of the manufacturer (QIAGEN). The rN proteins of PUUV strains Vranica/Hällnäs (PUUV-Vra), Kazan (PUUV-Kaz) and Sotkamo (PUUV-Sot), HTNV (strain Fojnica), SNV and ANDV were purified according to the same protocol.

Human Sera and Serum pools

An anti-SEOV human serum and two anti-HTNV human sera originated from the Republic of Korea. The anti-DOBV and anti-PUUV serum pools were each generated from four routine diagnostic serum samples of German HFRS patients regarded as positive for anti-DOBV-IgG and anti-PUUV-IgG, respectively, by in-house mAb-capture ELISA and Western blot tests based on yeast-expressed DOBV and PUUV rN proteins [Meisel et al., unpublished data] and chemiluminescence focus reduction neutralization assays [44]. The anti-SNV pool contained 15 routine diagnostic serum samples of HCPS patients from the US found to be anti-SNV-IgG positive by SNV strip blot assay [45] and our in-house SNV-IgG ELISA and Western blot tests using yeast-expressed SNV rN protein (J. Schmidt et al., submitted for publication]. For preparation of an anti-ANDV pool 56 anti-ANDV-IgG-positive serum samples derived from Argentinean and Chilean HCPS patients were used which were confirmed to be reactive by in-house SNV- or ANDV-IgG ELISA and Western blot tests [46,47, J. Schmidt et al., submitted for publication]. As a control a negative serum pool was generated from German serum samples of our routine diagnostics found to be negative for anti-HTNV-, anti-DOBV-, anti-PUUV-, anti-SNV- and anti-ANDV-IgG by our in-house ELISA tests based on the corresponding yeast-expressed rN proteins (29, Schmidt et al., submitted for publication].

Immunization of Rabbits with Yeast-expressed Hantavirus N Proteins

Three rabbits were immunized subcutaneously three times with an interval of 2–4 weeks with 100 μg of one of the purified His-tagged, yeast-expressed rN proteins of SEOV, SNV or ANDV dissolved in PBS. Blood samples were taken 1 week after booster immunization and up to 3 weeks later. After clotting of the blood and centrifugation the sera were stored at −20°C until use. The generation of HTNV- and PUUV-Sot-rN protein-specific rabbit sera followed the same protocol as has been described recently [29].

SDS-Polyacrylamide Gel Electrophoresis (SDS–PAGE) and Western Blot

SDS–PAGE and Western blot analysis were performed as described recently [29]. Aliquots of 2 μg of each purified rN protein of SEOV, ANDV, SNV, PUUV-Vra, PUUV-Sot, PUUV-Kaz and HTNV were run on a 12.5% SDS polyacrylamide gel and electro-blotted to nitrocellulose membrane. After blocking, the membranes were incubated overnight at room temperature (RT) with a panel of N-specific monoclonal antibodies (mAbs)

(diluted 1/50 up to 1/1000) raised against SEOV (mAb R31; purchased from Progen Biotechnik GmbH, Heidelberg, Germany), HTNV (mAb B5D9, [48]; from Progen Biotechnik GmbH; mAbs E5/G6, Eco2, [49]), PUUV (mAb A1C5, [48]; from Progen Biotechnik GmbH; mAbs 1C12, 5E1, 3G5, 5A3, 2E12, 4C3, [50]), SNV (mAb 5F1/F7) and ANDV (mAb 5C2/E10; both from Immunological and Biochemical Testsystems GmbH, Reutlingen, Germany). A His-tag-specific mAb was purchased from Amersham Pharmacia Biotech (Freiburg, Germany). The filters were washed three times and incubated for 4 h at RT with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Sigma, Deisenhofen, Germany) diluted 1/250 in serum dilution buffer. Finally the filters were stained with 4-chloro-1-naphthol substrate prepared according to the protocol of the manufacturer (Sigma).

ELISA

The ELISA investigations were performed essentially as described recently [29]. Briefly, polystyrene microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight with 2 μg/ml SEOV rN protein (or the rN proteins of HTNV, PUUV-Vra, PUUV-Sot, ANDV and SNV) diluted in carbonate buffer. After blocking with 3% bovine serum albumin (BSA) in PBS containing 0.05% Tween 20 100 μl of 1/200 diluted rat or rabbit sera, or 1/400 diluted human serum in 1% BSA in PBS containing 0.05% Tween 20 were added. After an incubation of 2 h to each well 100 μl HRP-conjugated goat anti-rat IgG (Sigma; dilution of 1/5000) or anti-rabbit IgG (Sigma; dilution of 1/3000) or anti-human IgG (DakoCytomation, Hamburg, Germany; diluted 1/6000) were added, and the plates were incubated again at 37°C for 1 h. After 10 min of incubation with 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) (Biorad, Hercules, CA) at RT the reaction was stopped by addition of 100 μl of 1 M H_2SO_4. Finally, the optical density (OD) values were measured at 450 nm (reference 620 nm).

The final OD value for each serum sample was calculated as the difference of the OD values measured in antigen-containing and antigen-free wells. These final OD values for serum dilutions of 1/400 or 1/200 were regarded as positive if the mean OD exceeded the mean OD + 3 standard deviations (SD) obtained with negative control samples. The endpoint titer for each serum sample was defined as the serum dilution where the OD value is three-times higher as the background OD value that is measured in highly diluted sera and does not decrease with further dilution of the serum. In our experiments the background OD varied between 0.01 and 0.1.

Results and Discussion

Sequence Analysis and Phylogeny of the N Protein-encoding Sequence of SEOV Strain 80-39

Comparison of the nucleotide sequence of the N protein-encoding open reading frame (ORF) from 80-39-Berlin (80-39-B), a cell culture-passaged Korean SEOV strain used herein, to the recently published corresponding sequence of SEOV strain 80-39 (AY273791) demonstrated only two silent nucleotide exchanges G15A (codon position 5) and G1275A (codon position 425). This results in a sequence identity of 99.6% at the nucleotide and 100% at the amino acid level (data not shown). As observed for all SEOV and HTNV sequences, in front of the N-ORF of SEOV 80-39-B two very close, in-frame ATG codons were found (data not shown). As expected for a Murinae-adapted virus, the potential second ORF on the S segment – present in almost all Arvicolinae- and Sigmodontinae-adapted hantaviruses – was found to lack a translation initiation codon and to be interrupted by numerous stop codons (data not shown). This very high identity of two cell culture-adapted lineages of SEOV strain 80-39 might suggest strong selection constraints in the Vero E6 cell culture. This is in line with the observation of a high genetic stability of SEOV maintained under a natural environment and sequence identities of SEOV strains isolated from various districts of Eastern Asia [51,52].

The nucleotide sequence diversity of SEOV 80-39-B to N protein-encoding sequences of other SEOV strains ranged from 1.3% for the most related (American) strain Tchoupitoulas (GenBank accession number AF329389) to 12.5% for the most ancestral SEOV strain Gou3 from China (GenBank accession number AB027522; see Fig. 1). As expected, the level of amino acid

Fig. 1. Topologies for a hantavirus phylogeny based on nucleocapsid (N) protein-encoding gene sequences. Maximum-likelihood (ML) strict consensus tree based on GTR + Γ + I model ($\alpha = 1.2286$, proportion of invariable sites = 0.2900). Neighbour-joining (NJ) based on LogDet/paralinear distances recovered the same topology. Values above branches refer to ML bootstrap proportions from 100 iterations, values below branches represent NJ bootstrap proportions from 1000 iterations. Asterisk indicates that the node was not supported by the reconstruction method. The SEOV branches are marked by light-grey background; the obtained sequence is indicated by an arrow. The following analyzed SEOV S segment sequences are included (accession numbers put in parentheses): SEOV strains 80-39 (AY273791), Tchoupitoulas (Tchou; AF329389), SR-11 (M34882), IR461 (AF329388), L99 (AF488708), R22 (AF488707), K24-v2 (AF288655), Hb8610 (AF288643), zy27 (AF406965), Pf26 (AY006465), Z37 (AF187082), Gou3 (AB027522). As outgroup the following sequences were used: HTNV strains 76-118 (M14626), A9 (AF329390), AH09 (AF285264); DOBV strains Esl/862Aa (AJ269550) and Slo/Af (L41916); SNV strain NM H10 (L25784), El Moro Canyon hantavirus (ELMCV) strain RM-97 (U11427), TULV strain Moravia/5302v/95 (Z69991), and PUUV strain Vranica/Hällnäs (U14137).

divergence between the SEOV strains was much lower (0.3% to SR-11 – 1.4% for IR461). The divergence of the SEOV sequence 80-39-B to sequences of the most closely related hantavirus species HTNV and DOBV is much higher ranging from about 25–27% at the nucleotide level to 15–18% at the amino acid level. The diversity reaches a highest value of about 40% at both the nucleotide and amino acid level for the most distantly related Arvicolinae- and Sigmodontinae-adapted viruses SNV, ELMCV, PUUV and TULV used as outgroup viruses (data not shown).

The results of various codon bias measures and the G + C content at different codon positions are listed in Table 1. ENC (Effective Number of Codons) is a very simple, but effective way of measuring codon use bias [34]. ENC ranges from 20, if only one codon is used for each amino acid, to 61, if all codons are used equally. ENC values of 35 or less are considered biased and genes with low ENC values are restricted in the use of synonymous codons compared with genes with high ENC values. In this study, a total of 22 N protein-encoding hantavirus sequences including 13 from SEOV strains have ENC values that are indicative of more random codon usage (ranging from 47.2% to 54.5%). Interspecific comparison of ENC values also revealed that there is minor variation in codon usage by the same gene in the different hantavirus genomes. Codon Bias Index (CBI) is a measure of the deviation from the equal use of synonymous codons [35]. CBI values range from 0 (uniform use

Table 1. Various measures of codon usage bias and GC content at different codon positions of each hantavirus N protein-encoding gene examined

Virus	Strain	ENC	CBI	SChi2	G + C2	G + C3s	G + Cc
SEOV	80-39-B	49.012	0.322	0.332	0.399	0.414	0.459
	80-39	49.058	0.318	0.330	0.399	0.419	0.461
	Tchou	48.912	0.324	0.325	0.399	0.414	0.459
	SR-11	48.865	0.322	0.330	0.401	0.421	0.462
	IR461	47.205	0.349	0.364	0.394	0.415	0.455
	L99	48.387	0.319	0.320	0.399	0.414	0.458
	R22	48.574	0.317	0.314	0.396	0.409	0.456
	K24-v2	48.681	0.322	0.322	0.396	0.416	0.459
	Hb8610	48.845	0.322	0.309	0.401	0.421	0.461
	zy27	48.187	0.346	0.340	0.401	0.419	0.462
	pf26	48.680	0.339	0.323	0.399	0.415	0.460
	Z37	47.826	0.328	0.344	0.401	0.421	0.462
	Gou3	50.579	0.351	0.329	0.401	0.428	0.468
HTNV	76-118	49.623	0.331	0.344	0.392	0.407	0.451
	A9	49.376	0.324	0.324	0.389	0.385	0.442
	AH09	51.511	0.300	0.294	0.392	0.417	0.450
DOBV	Esl/862Aa	54.480	0.281	0.228	0.382	0.396	0.446
	Slo/Af	50.725	0.286	0.273	0.380	0.432	0.458
SNV	NM H10	53.153	0.292	0.247	0.394	0.363	0.435
ELMCV	RM-97	48.977	0.353	0.323	0.380	0.350	0.425
TULV	Moravia/5302v/95	53.065	0.279	0.249	0.377	0.391	0.436
PUUV	Vranica/Hällnäs	48.307	0.334	0.323	0.358	0.341	0.421

ENC, effective number of codons [34]; CBI, codon bias index [35]; SChi2, Scaled Chi Square values [36]. (G + C2), (G + C3), (G + Cc), G + C content at the second, third and all coding positions, respectively, for each N protein-encoding sequence analysed. For details see Materials and Methods section.

of synonymous codons) to 1 (maximum codon bias). The CBI values listed in Table 1 are indicative of random codon usage and display a relatively low degree of variation in codon usage. The same is true for the SChi2 values (Table 1), another measure of potential codon bias based on the difference between the observed number of codons and those expected from equal usage of codons [36]. We also found that the percentage of GC-nucleotides of each of the 22 homologous genes examined in this study were very similar (ranging from 42.1% for PUUV to 46.8% for SEOV strain Gou3).

The reconstruction of the phylogenetic relationships of SEOV strains by maximum likelihood (ML) methods demonstrated that they represent a monophyletic group (Fig. 1). In line with previous data [52], the *R. rattus*-associated SEOV strain Gou3 represented the most ancestral strain, as evidenced also by phylogenetic investigation of M segment sequences (data not shown). As expected from the high-level of sequence diversity of Arvicolinae- and Sigmodontinae-adapted viruses SNV, ELMCV, PUUV and TULV (see above) these viruses form clearly separated branches in the ML tree. The same topology was inferred by using the NJ algorithm based on the LogDet/paralinear distances suitable for tree reconstruction where base compositions vary significantly between sequences (Fig. 1). The relationships in a tree based on codon usage similarities (data not shown) do not coincide with the relationships by virtue of similarity of nucleotide substitutions and therefore, we can be confident that our hypothesis of phylogenetic relationships is not being constructed because of problematic codon bias and nucleotide composition effects [53].

Expression in Yeast, Purification and Antigenic Characterization of SEOV rN Protein

As expected, the main portion of the yeast-expressed rN protein of SEOV strain 80-39-B was recovered from the nickel-chelation chromatography column in buffer E elution fractions (pH 4.5). The analysis

of these fractions in 12.5% SDS polyacrylamide gels revealed the presence of a protein band of the expected molecular mass of about 49 kDa (data not shown). The yield of the purified protein of about 0.9 mg/g wet weight corresponds to those values observed for rN proteins of PUUV-Vra [28] and other hantaviruses [29]. Analysis in the SDS-PAGE and immunoblot using a His-tag specific mAb demonstrated the absence of degradation products suggesting a highly pure and stable rN protein (data not shown).

As expected, the SEOV-specific mAb R31 reacted with the yeast-expressed SEOV rN protein. In addition, this mAb reacted also with rN protein of HTNV, but failed to recognize rN antigens of SNV, ANDV and PUUV strains (data not shown). In line with previous data [49], the antigenic similarity of rN proteins of SEOV and HTNV was also confirmed by the reactivity of HTNV-specific mAbs E5/G6 and Eco2 with the SEOV rN protein. The SEOV rN protein was also detected by the highly cross-reactive mAbs 1C12, 5A3 and 4C3 raised against PUUV confirming earlier data [54]. In contrast it did not react with the HTNV-specific mAb B5D9, the PUUV-specific mAbs 5E1, 3G5, 2E12 and AlC5, SNV-specific mAb 5F1/F7 and ANDV-specific mAb 5C2/E10 (data not shown). The discrepancies between the reactivity of mAb 2E12 with HTNV and SEOV in immunofluorescence assays [54] and its failure to react in Western blots with rN proteins of HTNV [29] and SEOV (this paper) is most likely caused by a discontinous nature of its epitope [50].

Cross-reactivity of Rabbit Sera Raised Against rN Proteins of SEOV and other Hantaviruses

The immunization of a rabbit with purified SEOV rN protein resulted in the induction of a high-titered SEOV-specific antibody response (Table 2). Similarly, rabbits immunized with yeast-expressed rN proteins of SNV and ANDV developed high titers of homologous antibodies (Table 2). The observed strong antibody response is in line with the immunogenicity of yeast-expressed rN proteins of other hantaviruses observed in rabbits [29, this paper] and mice [55].

As expected, all rabbit sera were found to react not only strongly with the homologous, but also with heterologous rN proteins. In general, the reciprocal endpoint titer for the homologous rN antigen was found to be the highest (Table 2; given in bold). The titer of N-specific antibodies in the SEOV rN-immunized rabbit was only slightly lower to the rN protein of the closely related HTNV, but much lower to those of PUUV-Sot, SNV and ANDV. Similarly, the anti-HTNV rN rabbit serum [29] reacted with equal titers to SEOV and HTNV rN protein (Table 2).

Analysis of the Kinetics of the Homologous and Cross-reactive Antibody Response of Experimentally SEOV-infected rats

In the first follow-up serum sample (taken 7 days after infection) of all four experimentally SEOV-infected rats [30] high-titered SEOV-N-specific antibodies were detected (Fig. 2). This is in line with data obtained for these rats using a recently developed ELISA based on *E. coli*-expressed HTNV rN protein [31].

In general, the reciprocal endpoint titers for all four rats at all time points for SEOV were higher than those for HTNV and PUUV-Vra. The level of cross-reactivity to HTNV rN protein was found to be increased during the time of observation reaching the highest endpoint titer in the latest follow-up serum sample. The level of cross-reactivity to PUUV-Vra rN protein was much lower (Fig. 2A, B and D) or even totally non-detectable (Fig. 2C). At 7 days post-infection in 3 of 4 rats HTNV and PUUV-Vra rN proteins failed to detect hantavirus-specific antibodies. These data are in line with observations demonstrating the necessity of a homologous N antigen for a highly sensitive detection of hantavirus-specific antibodies in humans, especially during the early phase of infection (46,56, J. Schmidt et al., submitted for publication]. As reported for human sera [57, J. Schmidt et al., submitted for publication], the level of cross-reactivity was more pronounced for rat sera taken during the later phase of infection.

Potential use of Yeast-expressed SEOV rN Protein for Diagnostic Purposes and Seroprevalence Studies

In general, the reciprocal endpoint titers of anti-SEOV, -HTNV, -DOBV, -SNV and -ANDV positive human sera or serum pools for the

Table 2. Cross -reactivity of hantavirus rN proteins with sera of rN-immunized rabbits

Serum of rabbit immunized with rN of	rN antigens used for the detection of lgG antibodies with ELISA				
	SEOV	HTNV	PUUV-Sot	SNV	ANDV
SEOV	**3,300,000**	1,600,000	76,800	38,400	51,200
HTNV	820,000	**820,000**	204,800	76,800	102,400
PUUV-Sot	6,600,000	6,600,000	**6,600,000**	3,300,000	6,600,000
SNV	3,300,000	3,300,000	13,200,000	**26,400,000**	13,200,000
ANDV	1,600,000	1,600,000	13,200,000	26,400,000	**52,800,000**

Given are the reciprocal endpoint titers. The homologous titers are shown in bold.

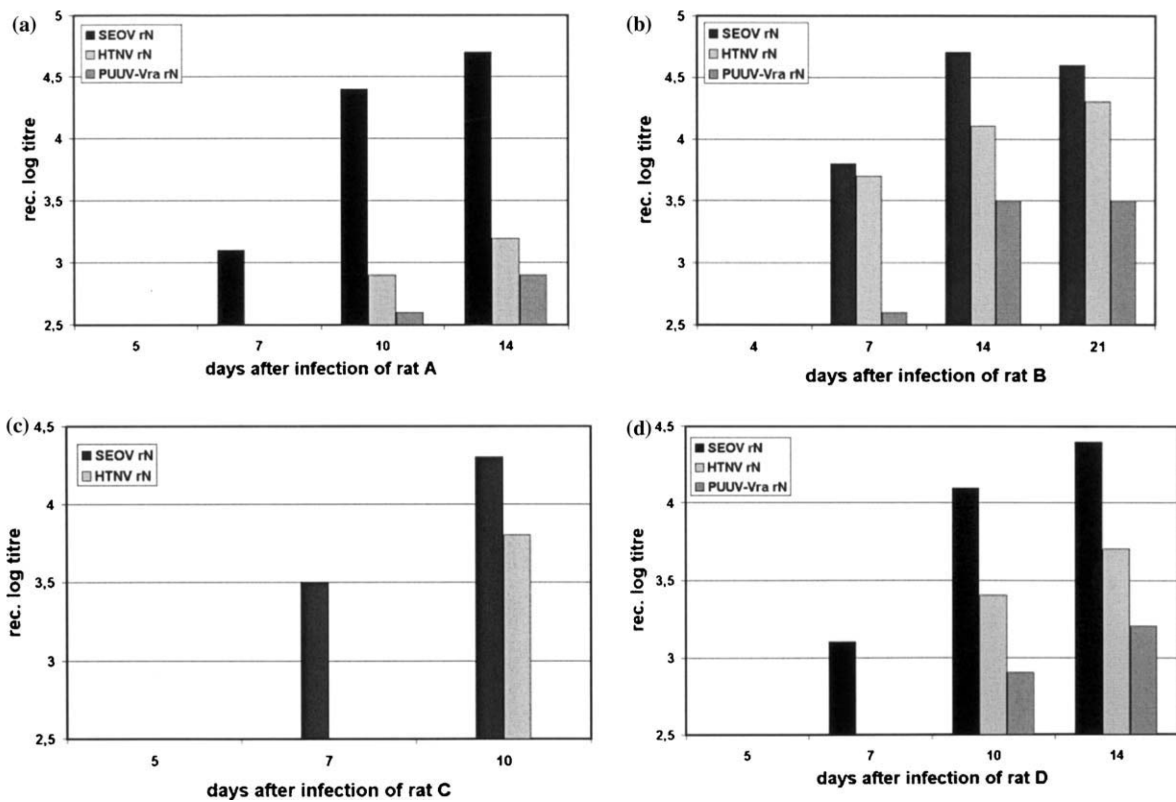

Fig. 2. ELISA reactivity of serial follow-up serum samples of four experimentally SEOV-infected Wistar rats using yeast-expressed rN proteins of SEOV, HTNV and PUUV-Vra. Microtiter plates were coated with rN proteins of SEOV (strain 80-39-B), HTNV (strain Fojnica) or PUUV (strain Vranica/Hällnäs). Thereafter, rat sera diluted serially two fold with an initial dilution of 1/200 were added. After incubation with HRP-labelled anti-rat IgG conjugate the immune reaction was visualized by addition of TMB substrate. Given are the reciprocal endpoint titers. The initial reciprocal log titer of 2.5 corresponds to a dilution of 1/400. As negative control, a serum from a wild-trapped rat from Japan, previously demonstrated to be non-infected [31], was used.

respective homologous antigens were found again to be the highest (Table 3). Thus, the anti-SEOV-positive serum reacted to the homologous SEOV rN antigen with the highest endpoint titer. As expected, its reactivity with HTNV rN was lower and the serum failed to react with rN proteins of the more distantly related PUUV, SNV and ANDV.

In contrast anti-HTNV- and anti-DOBV-positive serum pools reacted to equal endpoint titers to

Table 3. Cross-reactivity of hantavirus rN proteins with serum pools of HFRS patients infected with SEOV, HTNV, DOBV or PUUV and HCPS patients infected with SNV or ANDV, respectively

Human sera or serum pools	rN antigens used for the detection of IgG antibodies with ELISA				
	SEOV rN	HTNV rN	PUUV-Vra rN	SNV rN	ANDV rN
Anti-SEOV	25,600	4,800	<400	<400	<400
Anti-HTNV	51,200	51,200	2,400	2,400	3,200
Anti-DOBV	25,600	25,600	1,600	<400	<400
Anti-PUUV	9,600	1,600	25,600	12,800	12,800
Anti-SNV	<400	<400	4,800	51,200	12,800
Anti-ANDV	6,400	1,600	12,800	12,800	51,200

Given are the reciprocal endpoint titers. The highest titers are given in bold.

both SEOV and HTNV rN proteins. Pools of anti-PUUV and anti-ANDV-positive sera were found to cross-react also with SEOV rN protein, whereas the anti-SNV-positive pool failed to react with SEOV rN protein (Table 3). Interestingly, the level of cross-reactivity of the anti-PUUV- and anti-ANDV-positive pools to the HTNV rN protein was lower compared to that to SEOV rN antigen; the anti-SNV serum pool failed to react with HTNV rN protein. This difference in the cross-reactivity of anti-ANDV- and anti-SNV-positive serum pools with SEOV rN protein might be due to the fact that the anti-SNV-positive serum pool contained only acute phase sera whereas the anti-ANDV serum pool contained also late, convalescent sera.

Because of a previous report about a high seroprevalence of rats in Germany for SEOV [21] we performed an initial study on a small number of sera from wild rats (n = 11). These rats stemming from farms in Westphalia/Germany belonged to the species *R. norvegicus* as evidenced by the identity of the mt 12S rDNA sequence of liver tissue samples of two rats with that of an already published *R. norvegicus* sequence from Denmark (accession number AJ 428514; data not shown) and its divergency from the corresponding sequence of *R. rattus* (accession number AJ005780). As no SEOV-specific antibodies could be detected in this small number of wild-trapped rats (data not shown), additional studies are needed to prove the hantavirus seroprevalence of rats in Germany.

In conclusion, the N protein-encoding sequence of SEOV strain 80-39-B was expressed to high level in yeast *S. cerevisiae*. Initial studies suggest the usefulness of the antigen for the establishment of ELISAs to detect SEOV infections in rat and human sera.

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References

1. Schmaljohn C.S. and Nichol S.T. (eds). *Hantaviruses. Curr Top Microbiol Immunol 256*. Springer, Berlin, Heidelberg, New York, 2001.
2. Krüger D.H., Ulrich R., and Lundkvist Å., Microbes Infect *3*, 1129–1144, 2001.
3. Lee H.W., Lee P.W., and Johnson K.M., J Infect Dis *137*, 298–308, 1978.
4. Lee H.W., Rev Infect Dis *11*, S864–S876, 1989.

5. Lee H.W., Baek L.J., and Johnson K.M., J Infect Dis *146*, 638–644, 1982.
6. Kim Y.S., Ahn C., Han J.S., Kim S., Lee J.S., and Lee P.W., Nephron *71*, 419–427, 1995.
7. Lokugamage K., Kariwa H., Hayasaka D., Zhong Cui B., Iwasaki T., Lokugamage N., Ivanov L.I., Volkov V.I., Demenev V.A., Slonova R., Kompanets G., Kushnaryova T., Kurata T., Maeda K., Araki K., Mizutani T., Yoshimatsu K., Arikawa J., and Takashima I., Emerg Infect Dis *8*, 768-776, 2002.
8. Kariwa H., Fujiki M., Yoshimatsu K., Arikawa J., Takashima I., and Hashimoto N., Arch Virol *143*, 365–374, 1998.
9. Hinson E.R., Shone S.M., Zink M.C., Glass G.E., and Klein S.L., Am J Trop Med Hyg *70*, 310–317, 2004.
10. Liang M., Li D., Xiao S.Y., Hang C., Rossi C.A., and Schmaljohn C.S., Virus Res *31*, 219–233, 1994.
11. Kariwa H., Yoshimatsu K., Araki K., Chayama K., Kumada H., Ogino M., Ebihara H., Murphy M.E., Mizutani T., Takashima I., and Arikawa J., Microbiol Immunol *44*, 357–362, 2000.
12. Arikawa J., Yoshimatsu K., and Kariwa H., Jpn J Infect Dis *54*, 95–102, 2001.
13. Yashina L.N., Patrushev N.A., Ivanov L.I., Slonova R.A., Mishin V.P., Kompanez G.G., Zdanovskaya N.I., Kuzina I.I., Safronov P.F., Chizhikov V.E., Schmaljohn C., and Netesov S.V., Virus Res *70*, 31–44, 2000.
14. Groen J., Suharti C., Koraka P., van Gorp E.C., Sutaryo J., Lundkvist Å., and Osterhaus A.D., Infection *30*, 326–327, 2002.
15. Reynes J.M., Soares J.L., Hüe T., Bouloy M., Sun S., Kruy S.L., Flye Sainte Marie F., and Zeller H., Microbes Infect *5*, 769–773, 2003.
16. Plyusnin A., and Morzunov S.P., Curr Top Microbiol Immunol *256*, 47–75, 2001.
17. Glass G.E., Watson A.J., LeDuc J.W., and Childs J.E., Nephron *68*, 48–51, 1994.
18. Iversson L.B., da Rosa A.P., Rosa M.D., Lomar A.V., Sasaki Mda. G., and LeDuc J.W., Rev Assoc Med Bras *40*, 85–92, 1994.
19. Le Duc J.W., Smith G.A., Childs J.E., Pinheiro F.P., Maiztegui J.I., Niklasson B., Antoniadis A., Robinson D.M., Khin M., Shortridge K.F., Wooster M.T., Elwell M.R., Ilbery P.L.T., Koech D., Rosa E.S.T., and Rosen I., Bull World Health Org *64*, 139–144, 1986.
20. Korch G.W., Childs J.E., Glass G.E., Rossi C.A., and LeDuc J.W., Am J Trop Med Hyg *41*, 230–240, 1989.
21. Pilaski J., Ellerich C., Kreutzer T., Lang A., Benik W., Pohl-Koppe A., Bode L., Vanek E., Autenrieth I.B., Bigos K., and Lee H.W., Lancet *337*, 111, 1991.
22. McCaughey C., Montgomery W.I., Twomey N., Addley M., O'Neill H.J., and Coyle P.V., Epidemiol Infect *117*, 361–365, 1996.
23. Ibrahim I.N., Sudomo M., Morita C., Uemura S., Muramatsu Y., Ueno H., and Kitamura T., Jpn J Med Sci Biol *49*, 69–74, 1996.
24. Papa A., Mills J.N., Kouidou S., Ma B., Papadimitriou E., and Antoniadis A., Emerg Infect Dis *6*, 654–655, 2000.
25. Shi X., McCaughey C., and Elliott R.M., J Med Virol *71*, 105–109, 2003.
26. Ulrich R., Hjelle B., Pitra C., and Krüger D.H., Intervirology *45*, 318–327, 2002.
27. Sjölander K.B., Elgh F., Kallio-Kokko H., Vapalahti O., Hagglund M., Palmcrantz V., Juto P., Vaheri A., Niklasson B., and Lundkvist Å., J Clin Microbiol *35*, 3264–3268, 1997.
28. Dargeviciute A., Brus Sjölander K., Sasnauskas K., Krüger D.H., Meisel H., Ulrich R., and Lundkvist Å., Vaccine *20*, 3523–3531, 2002.
29. Razanskiene A., Schmidt J., Geldmacher A., Ritzi A., Niedrig M., Lundkvist Å., Krüger D.H., Meisel H., Sasnauskas K., and Ulrich R., J Biotechnol *111*, 319–333, 2004.
30. Yoshimatsu K., Arikawa J., Yoshida R., Li H., Yoo Y.-C., Kariwa K., Hashimoto N., Kakinuma M., Nobunaga T., and Azuma I., Lab Anim Sci *45*, 641–646, 1995.
31. Takakura A., Goto K., Itoh T., Yoshimatsu K., Takashima I., and Arikawa J., Exp Anim *52*, 25–30, 2003.
32. Rozas J. and Rozas R., Bioinformatics *15*, 174–175, 1999.
33. Sharp P.M., Tuohy T.M.F., and Mosurski K.R., Nucleic Acids Res *14*, 5125–5143, 1986.
34. Wright F., Gene *87*, 23–29, 1990.
35. Morton B.R., J Mol Evol *37*, 273–280, 1993.
36. Shields D.C., Sharp P.M., Higgins D.G., and Wright F., Mol Biol Evol *5*, 704–716, 1988.
37. McInerney J.O., Bioinformatics *14*, 372–373, 1998.
38. Xia X. and Xie Z., J Hered *92*, 371–373, 2001.
39. Hall T.A., Nucl Acids Symp Ser *41*, 95–98, 1999.
40. Swofford D.L., *PAUP*: Phylogenetic Analysis using Parsimony (*and Other Methods)*. Sinauer Associates, Sunderland, Mass., 2001.
41. Lockhart P.J., Steel M.A., Hendy M.D., and Penny D., Mol Biol Evol *11*, 605–612, 1994.
42. Nylander J.A.A., MrModeltest v1.0b. Program distributed by the author. Department of Systematic Zoology, Uppsala University, 2002. http://www.ebc.uu.se/systzoo/staff/nylander.htlm
43. Rodrigez F., Oliver J.F., Marin A., and Medina JR, J Theor Biol *142*, 485–501, 1990.
44. Heider H., Ziaja B., Priemer C., Lundkvist Å., Neyts J., Krüger D.H., and Ulrich R., J Virol Methods *96*, 17–23, 2001.
45. Hjelle B., Jenison S., Torrez-Martinez N., Herring B., Quan S., Polito A., Pichuantes S., Yamada T., Morris C., Elgh F., Lee H.W., Artsob H., and Dinello R., J Clin Microbiol *35*, 600–608, 1997.
46. Padula P.J., Rossi C.M., Della Valle M.O., Martinez P.V., Colavecchia S.B., Edelstein A., Miguel S.D.L., Rabinovich R.D., and Segura E.L., J Med Microbiol *49*, 149–155, 2000.
47. Rossi C. and Ksiazek T., In: Lee H.W., Calisher C., and Schmaljohn C.S. (eds). *Manual of Hemorrhagic Fever with Renal Syndrome and Hantavirus Pulmonary Syndrome*. WHO Collaborating Center for Virus Reference and Research, Seoul, 1999, pp. 87–91.
48. Zöller L.G., Yang S., Gött P., Bautz E.K., and Darai G., J Clin Microbiol *31*, 1194–1199, 1993.

49. Yoshimatsu K., Arikawa J., Tamura M., Yoshida R., Lundkvist Å., Niklasson B., Kariwa H., and Azuma I., J Gen Virol *77*, 695–704, 1996.
50. Lundkvist Å., Meisel H., Koletzki D., Lankinen H., Cifire F., Geldmacher A., Sibold C., Gött P., Vaheri A., Krüger D.H., and Ulrich R., Viral Immunol *15*, 177–192, 2002.
51. Kariwa H., Isegawa Y., Arikawa J., Takashima I., Ueda S., Yamanishi K., and Hashimoto N., Virus Res *33*, 27–38, 1994.
52. Wang H., Yoshimatsu K., Ebihara H., Ogino M., Araki K., Kariwa H., Wang Z., Luo Z., Li D., Hang C., and Arikawa J., Virology *278*, 332–345, 2000.
53. He M. and Haymer D.S., J Mol Evol *41*, 141–149, 1995.
54. Dzagurova T., Tkachenko E., Slonova R., Ivanov L., Ivanidze E., Markeshin S., Dekonenko A., Niklasson B., and Lundkvist Å., Arch Virol *140*, 1763–1773, 1995.
55. Geldmacher A., Schmaler M.,Krüger D.H., and Ulrich R., Viral Immunol *17*, 115–122, 2004.
56. Sjölander K.B., and Lundkvist Å., J Virol Methods *80*, 137–143, 1999.
57. Elgh F., Linderholm M., Wadell G., Tarnvik A., and Juto P., FEMS Immunol Med Microbiol *22*, 309–315, 1998.