(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

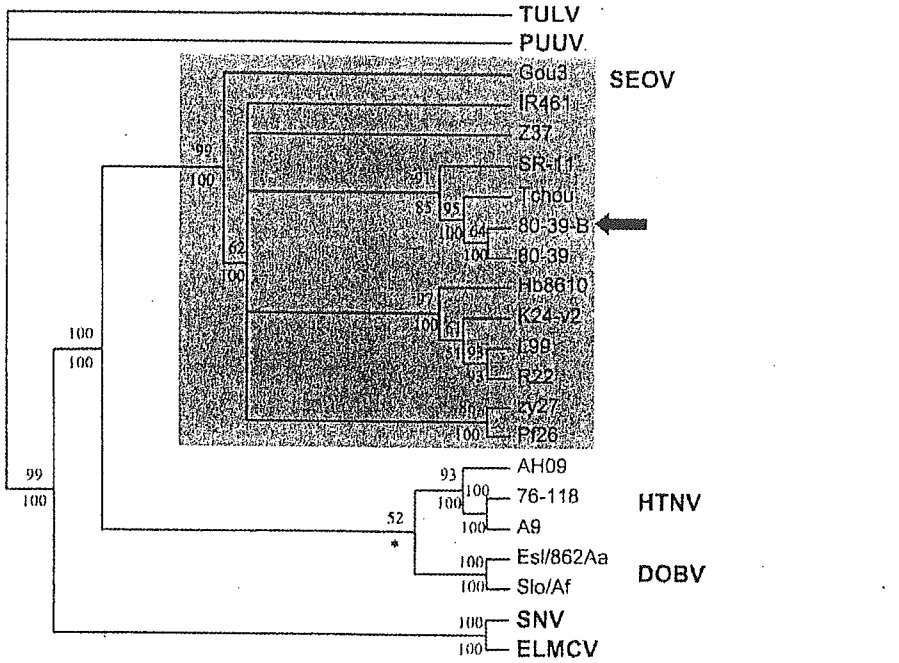

Fig. 1. Topologies for a hantavirus phylogeny based on nucleocapsid (N) protein-encoding gene sequences. Maximum-likelihood (ML) strict consensus tree based on GTR + Γ + I model (α = 1.2286, proportion of invariable sites = 0.2900). Neighbour-joining (NJ) based on LogDet/paralinear distances recovered 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 IgG 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.

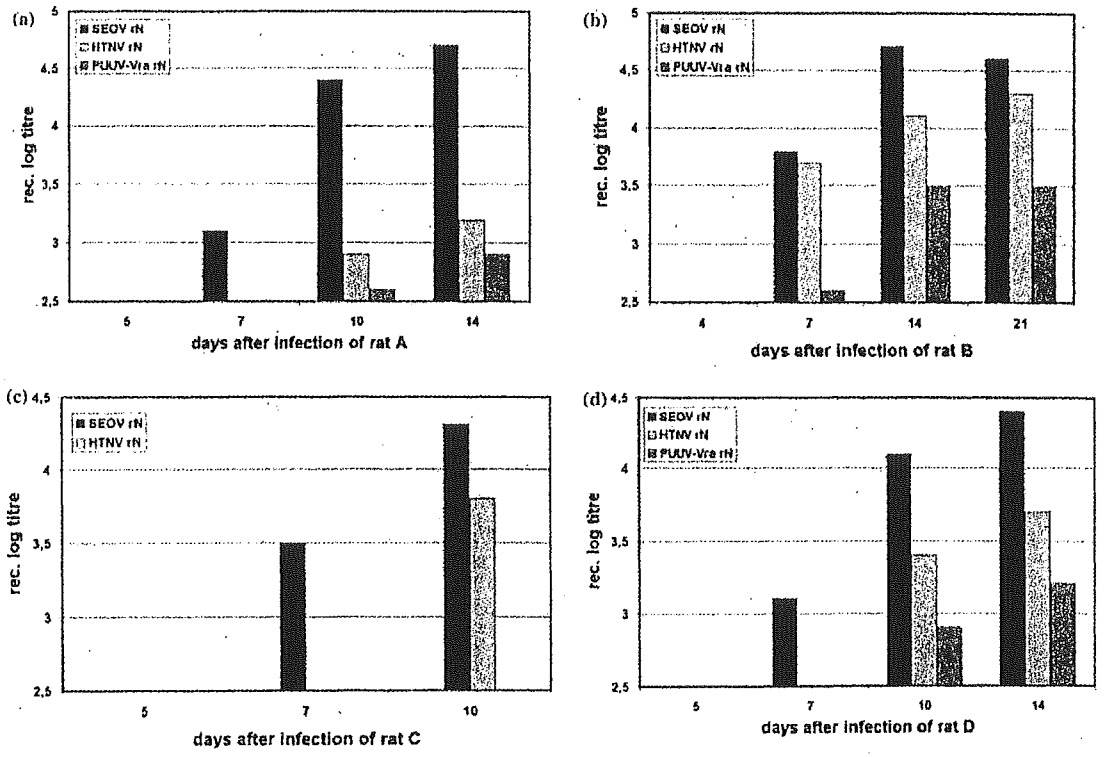

Fig. 2. ELISA reactivity of serial follow-up serum samples of four experimentally SEOV-infected Wistar rats using yeast-expressed rN proteins of SEOV, HTNV and PUUV-Vra. Microtiter plates were coated with rN proteins of SEOV (strain 80-39-B), HTNV (strain Fojnica) or PUUV (strain Vranica/Hällnäs). Thereafter, 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

	rN antigens used for the detection of IgG antibodies with ELISA				
Human sera or serum pools	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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A pilot study for serological evidence of hantavirus infection in human population in south India

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***Background & objectives*: Hantaviruses are rodent-borne viruses of the family *Bunyaviridae* that have been identified as aetiological agents of two human diseases, haemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). There are no reports of hantavirus infections in humans from India, hence this pilot study was undertaken to provide the serological evidence of hantavirus infections in humans in south India.**

***Methods*: Serum samples were obtained from individuals with acute febrile illness and from voluntary blood donors, majority of whom were from south India. Serum samples were tested for anti-hantavirus IgM using a commercial enzyme immunoassay (EIA). Samples found positive by the EIA were tested by an indirect immunofluorescence assay (IFA) using slides coated with Seoul virus (SEOV) infected cells as substrate.**

***Results*: Of the 152 serum samples from individuals with pyrexic illness, 23 (14.7%) were positive for anti-hantavirus IgM by EIA. In contrast, only 5.7 per cent of healthy blood donors were positive by this assay. Eighteen of the 22 (82%) EIA-positive samples from patients were positive by the IFA assay. In contrast, only 2 of the 5 (40%) blood donor EIA positive samples were positive in the IFA assay.**

***Interpretation & conclusion*: The finding of this study indicated the possible presence of hantavirus infections in the human population of India presenting both as asymptomatic and symptomatic infections. Further studies need to be done to confirm the findings on a larger sample using molecular techniques.**

Key words Enzyme immunoassay - hantavirus - IgM antibodies - pilot study

Hantaviruses are enveloped viruses with a negative-sense single stranded RNA genome and belong to the family *Bunyaviridae*[1]. The spectrum of clinical symptoms caused by hantaviruses in humans varies from sub-clinical presentation to severe haemorrhagic fever with renal syndrome (HFRS) or pulmonary syndrome (HPS). Several genotypes/serotypes have been described of which at least five are pathogenic to humans[2]. The vast majority of human hantavirus infections are asymptomatic[3].

The natural reservoirs of hantaviruses are small rodents and transmission to man is believed to occur via aerosolized excretions. The worldwide distribution of rodents known to harbour hantaviruses suggests great disease causing potential[4].

The clinical diagnosis of hantavirus infections has routinely been confirmed by immunofluorescence antibody assay (IFA) or enzyme-linked immunosorbent assays (ELISA)[5]. The presence of cross reacting antibodies makes it difficult to distinguish hantavirus species by serology. Molecular tests based on nested reverse transcriptase-polymerase chain reaction (RT-PCR) have been used for diagnosis. Primary isolation of hantaviruses is most often attempted using Vero E6 cell line in laboratories with biosafety level-3 (BSL-3) facilities[6].

Hantavirus nucleocapsid protein (N) antigen elicits a strong humoral response in infected patients and immunized animals. High levels of antibody to the N antigen have been detected which indicated that it could be suitable as the sole antigen for serodiagnosis[7].

The Thottapalayam virus, which belongs to the same family, was isolated from the spleen of a shrew captured in July 1964, in Vellore, North Arcot district, Tamil Nadu, India[8]. Subsequently there have been no studies showing hantavirus infections in India. We undertook this study to investigate for the serological evidence of hantavirus infections in the human population by detecting anti-hantavirus IgM antibodies.

Material & Methods

Blood samples were obtained from 152 individuals who had acute febrile illness (duration <14 days). These patients were categorized into three different categories: (*i*) patients (n=67) with dengue-like illness but negative for dengue serology by a commercial Dengue Duo IgM and IgG Rapid Strip test[9]; (*ii*) patients (n=51) with leptospirosis-like illness, but negative for leptospira serology by the microscopic agglutination test (MAT)[10]. These patients were included because the early signs and symptoms of hantavirus infections mimic that of dengue and leptospirosis[11]; and (*iii*) patients (n=34) with suspected hantavirus infection. The samples were included by convenient sampling and had been submitted to the laboratory for various tests requested by the clinicians. Serum samples were stored at -20°C until testing. All the tests were conducted at the Department of Clinical Virology, Christian Medical College, Vellore. Since this was a pilot study, sample size was not calculated. All these patients presented with complaints of high grade fever with chills and rigors of <14 days duration associated with myalgia, headache and haemorrhagic manifestations like petechiae and purpuric skin rash.

In addition, 87 blood samples from voluntary blood donors were used as controls for the study. Sixteen samples were also included from patients with other viral infections as shown by corresponding IgM testing including dengue (n=10) and cytomegalovirus (n=6) (by a commercial Euroimmun kit, Germany) to serve as disease controls. All study samples were collected during a period of 15 months (July 2002 to September 2003). This study protocol was approved by the institutional research committee for scientific content and ethics.

The hantavirus IgM ELISA kit was procured from Focus Technologies (USA). This kit has been certified by ISO for human testing. This is an indirect antibody detection system; the assays were performed according to the manufacturer's instructions. As per the manufacturer's claim, this assay detects IgM antibodies against most of the known hantavirus subtypes including Puumala (PUUV), Hantaan (HNTV), Seoul (SEOV), Sin Nombre (SNV) and Dobrava (DOBV).

Briefly, microwell plates were coated with a mixture of baculovirus-derived recombinant nucleocapsid proteins from hantavirus strains. Serum samples (1:101 dilution) were incubated for 60 min, and after washing, anti-human IgM-horse radish peroxidase conjugate was added to the wells and incubated for 30 min. Tetramethylbenzidine was used as a substrate. After adding the stop solution, the resultant colour change was quantified by an EIA reader ELx 800 (Bio-Tek Instruments Inc. Vermont, USA). The optical density (OD) is shown to be directly proportional to the amount of antigen- specific IgM present in the sample. Sample OD readings were compared with reference cut off OD readings. Results

were reported as index values relative to the 'cut off' calibrator. To calculate the index values, specimen OD values were divided by the mean of the 'cut off' calibrator OD values.

All the samples found positive by ELISA were further tested by an indirect immunofluorescence assay (IFA) using Seoul virus (SEOV) (strain SR-11) infected Vero E6 cells as substrate. The serum samples were tested at a dilution of 1:10. Serum samples (15 µl) were added onto the 24 well slides and incubated for 30 min. The slides were then washed three times with phosphate-buffered saline (PBS, *p*H=7.4) and incubated with polyclonal rabbit anti-human IgM FITC-labeled conjugate (Dakocytomation) for 30 min. The slides were then washed and after mounting it with buffered alkaline glycerol, were read under the fluorescence microscope. All spots wherein 50 per cent of infected cells showing a characteristic apple green cytoplasmic fluorescence were recorded as reactive. Samples showing a positive reaction at 1:10 dilution were further diluted and tested at dilutions 1:20, 1:40, 1:80.

Data were analysed by Chi-square test using the EPI INFO (Version 6.04b), $P<0.05$ was considered significant.

Table I. Samples positive by ELISA and immunofluorescence assay (IFA)

Status of sample	No. positive by IgM ELISA	No. (%) positive by IFA
Samples from suspected hantavirus cases	22	18 (86)*
Dengue positive sample	1	0
Voluntary blood donors	5	2 (40)

*Only 21 samples tested

Table II. Clinical and laboratory findings in individuals with pyrexia of unknown origin and positive for hantavirus IgM antibody (n=12)

Clinical signs and symptoms	No. (%) positive
Fever	12 (100)
Nausea and vomiting	7 (58)
Hepatomegaly	5 (42)
Myalgia and headache	5 (42)
Cough	4 (33)
Dyspnoea	2 (17)
Urinary symptoms*	2 (17)
Rash	1 (8)
Laboratory findings	*No. (%)*
Thrombocytopaenia**	8 (67)
Leucocytosis***	7 (58)
Elevated liver enzymes·	3 (25)

*Increased frequency of micturition, dysuria, flank pains and haematuria

<100000 cells/mm^3, * >9500 cells/mm^3
·SGPT, SGOT > 100 U/ml, Alkaline phosphatase

Results

A total of 255 serum samples were tested for hantavirus IgM by ELISA; 28 (10.98%) of these were positive for hantavirus IgM by EIA (Table I). Twenty seven (96.4%) of the 28 positive samples were retested and produced reproducible results; one sample was insufficient for retesting. Ten of 67 (14.9%) samples negative for dengue, 9 of 51 (17.6%) negative for leptospirosis, 3 of 34 (8.8%) of those with suspected clinical hantavirus infection, and 1 of 10 positive for dengue infection, were hantavirus IgM EIA positive. Of the 87 voluntary blood donors, 5 (5.7%) were hantavirus IgM EIA positive. None of the six samples positive for CMV serology was hantavirus IgM EIA positive.

Of the 28 hantavirus IgM positive samples, only 27 could be tested by the IFA. One sample was insufficient for testing. Of these, 20 (71.4%) were also positive by IFA. A representative number (n=5) of EIA negative samples were negative by IFA.

Examination of the patients' clinical records revealed that of the 23 patients who were seropositive for hantavirus IgM, eight had other underlying diseases. Records of three patients were not available for evaluation. The remaining 12 labeled as cases of pyrexia of unknown origin (PUO) were evaluated based on the clinical history at the time of presentation, the laboratory findings and the final diagnosis (Table II).

Among the individuals with febrile illnesses (n=152, 59.6%), 14.5 per cent (95% CI: 9.5-20.75) were positive for anti-hantavirus IgM antibodies. The difference in the rate of positives between the study group and the blood donor healthy control group (n=87)

where the positivity rate was 5.7 per cent (95% CI: 2.13-12.27) was statistically significant ($P<0.05$).

Discussion

This is probably the first study in India showing serological evidence of hantavirus infection in the Indian population. Clinically, the febrile illness produced by dengue virus, leptospira serotypes and hantaviruses may be indistinguishable[11]. The rate of IgM positive status in the febrile illness group was significantly higher than that seen in the healthy blood donors. We also found that this test did not pick up significant false positives in the samples positive for dengue or CMV IgM. It could be assumed that the positives in the control group indicated asymptomatic infections. Likewise, the hantavirus IgM seropositives in patients with other underlying diseases could be due to serological cross reactivity or asymptomatic infections. Alternatively, it could also be speculated that there are multiple hantaviruses in circulation and that at least one is of low pathogenicity.

It was noted that the mean of index values of the blood donor controls which were positive for hantavirus IgM (1.2±0.35) was lower than that of the cases (3.5±2.5). Such information could be used to establish a baseline 'cut off' index value to differentiate symptomatic from asymptomatic cases following screening of a larger number of individuals.

Hantavirus infections can appear clinically uncharacteristic and may mimic other syndromes. This compounds the difficulties in diagnosing hantavirus infections in areas where the disease is not endemic and clinical cases may be sporadic[12]. Further, less pathogenic hantaviruses may cause a greater amount of asymptomatic infections, as seen for HFRS in Europe and Asia[13].

Though a good association was seen in the findings of the two serological assays, EIA and IFA, IFA was cumbersome and required specialized equipment and experienced personnel for accurate interpretation. The difference in positives between the two assays could be due to the fact that the EIA had a cocktail of baculovirus-derived recombinant nucleocapsid antigens from different hantavirus strains while in the IFA only Seoul virus antigen was used. The EIA offered the advantage of a rapid and less complicated detection system and could be done even in middle-level laboratories.

Positive serology should be interpreted very cautiously when done on single serum samples. Paired serum samples may help delineating true hantaviral infections. Further, the results of serology should be correlated with clinical features to arrive at a definitive diagnosis. Future studies need to be focused on confirmatory testing by detecting the hantaviral genome using RT-PCR in samples collected in the acute phase of illness.

Since no previous published data on hantavirus infections in India are available and not much is known about laboratory diagnosis or clinical cases, general physicians are probably unaware of diseases with hantavirus aetiology. This suggests that there could be circulation of the hantaviruses in the Indian population. Further studies need to be done to identify hantaviruses in clinical samples by molecular techniques to definitely describe the clinical picture of hantaviral infections in India. The molecular techniques will include primers that can identify Thottapalayam virus, the only hantavirus isolate from India. These techniques will also help to identify the rodent reservoirs and to elucidate the importance of potentially unknown hantaviruses in India.

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