and foci were enumerated. Vero E6 cells infected with DTK/Ufa-97 exhibited small plaques (average diameter=1 mm to 2 mm) at 14 days post-infection, whereas cells infected with the PUUV Sotkamo strain did not form plaques (data not shown). In addition, the DTK/Ufa-97 foci (average diameter ± S.D., 196 μm±46 μm) were more than twice as large as the Sotkamo foci (average diameter ± S.D., 80 μm±6 μm) (data not shown).

Viral RNA copy number and virus titer

The DTK/Ufa-97-infected Vero E6 cells and the corresponding culture media were collected at various intervals after infection, and the viral RNA copy number and viral titer were determined. Viral RNA expression remained unchanged in Vero E6 cells grown in MEM, with approximately 1 × 10^3 to 7×10^3 copies of viral RNA transcribed during the 24 hr following infection. The copy number increased to approximately 5×10^4 copies at 3 dpi and remained at that level until 21 dpi (Fig. 1). In comparison, Sotkamo RNA replication occurred more slowly, reaching a plateau level at 7 dpi. When SFM was used instead of MEM, the DTK/Ufa-97 RNA copy number increased to 2.4× 10^5 by 14 dpi and remained at that level until 21 dpi. However, fewer than 4×10^3 copies of Sotkamo RNA were present at 21 dpi.

When Vero E6 cells were grown in MEM, the DTK/Ufa-97 viral titer reached a peak of 2.8×10^5 ffu/ml at 7 dpi, then decreased to 1.4×10^4 ffu/ml at 10 dpi and remained unchanged until 21 dpi (Fig. 2). In contrast, the Sotkamo titer reached a peak of 2.1×10^4 ffu/ml at 7 dpi, then declined to 6.0 × 10^3 ffu/ml at 10 dpi and remained unchanged until 21 dpi. When Vero E6 cells were grown in SFM, the DTK/Ufa-97 titer reached peaks of 4.5×10^4, 8.3×10^4, and 9.3×10^4 ffu/ml at 7, 14, and 21 dpi,

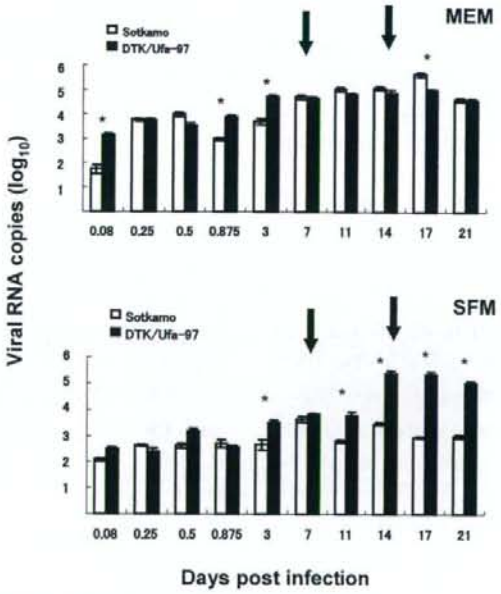

Fig. 1. Copy numbers of PUUV RNA in infected Vero E6 cells.
Cells were infected with PUUV strains Sotkamo and Ufa-97 at a multiplicity of infection (MOI) of 0.02, and were then maintained in MEM containing 10% FBS (*MEM*) or in *SFM*. Viral RNA copy numbers were determined via quadruplicate real-time PCR experiments. The number of viral RNA copies in infected cells is expressed as log10 of the RNA copy number. The standard deviation for each virus is shown at the top of the column corresponding to RNA copy number. Arrows indicate times when the medium was changed. Asterisks indicate significant differences, as determined using Student's t-tests (defined by $P < 0.05$).

respectively, whereas the Sotkamo titer remained less than 3.1×10^3 ffu/ml throughout the observation period.

Sequencing of the complete DTK / Ufa-97 genome

The full-length S, M, and L segments of the DTK/Ufa-97 genome were sequenced, and the resulting data were deposited into the DNA Data Bank of Japan under accession numbers AB 297665, AB297666, and AB297667, respectively. To our knowledge, this is the first complete genomic sequence of a human PUUV isolate from Russia. The S, M, and L segments of the DTK/Ufa-97 strain were 1,829, 3,682, and 6,550 nts in length, respectively (data not shown). These sequences differ from those of the Sotkamo genome only in the length of the S segment, which was 1 nt longer in Sotkamo strain (data not shown).

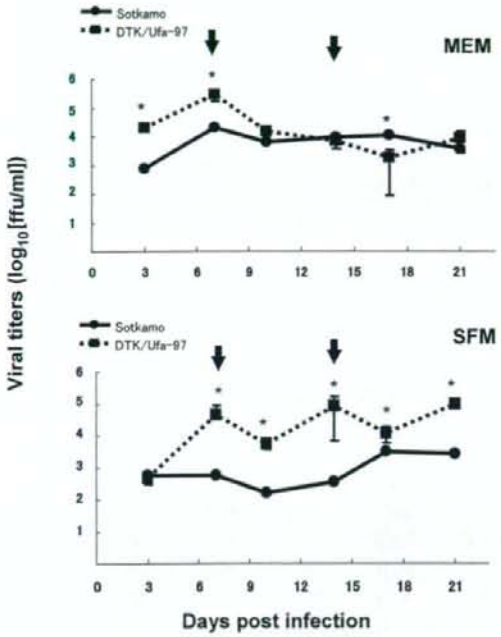

Fig. 2. Growth of PUUV strains.
Vero E6 cells were infected with Sotkamo or DTK/Ufa-97 at an MOI of 0.02 and maintained in either MEM or SFM. Supernatants were collected at the indicated times. The viral titer in the supernatant fraction was expressed as log_{10} [ffu/ml], and titers for each virus were determined in IFA triplicates. The standard deviation for each virus is shown at the top of the viral titer column. Arrows indicate the time points at which the medium was changed. Asterisks indicate significant differences, as determined using Student's t-tests (defined by $P < 0.05$).

Amino acid and nucleotide sequence comparisons

The nucleotide sequence of the DTK/Ufa-97 S segment was 99.9%, 100%, 99.5%, 99.5%, 93.9%, 93.9%, and 85.3% identical to the sequences of PUUV strains CG1820, P360, K27, CG17, Fs808, Kazan, and Sotkamo, respectively. Nucleotide sequence identity was greater than 93% among the Russian PUUVs; however, the Russian and Finnish PUUV Sotkamo strains were approximately 84% identical (Table 1). The predicted amino acid sequences of all of the PUUVs, including the Sotkamo strain, were more than 96% identical (Table 1).

The nucleotide sequence of the DTK/Ufa-97 M segment was 99.6%, 99.8%, 99.8%, 99.4%, 85.8%, 82.8%, and 80.3% identical to those of PUUV strains CG1820, P360, K27, CG17, Kazan, Sotkamo, and Umea/hu, respectively (Table 2). The M segments of the Bashkirian viruses, including DTK/Ufa-97, were more than 99% identical at the nucleotide level, whereas the M segments of the Bashkirian and Northern European strains were approximately 80.3% to 83.2% identical. The Russian PUUV M segments were more than 94.5% identical at the amino acid level, whereas the entire group of PUUV M segments were more than 88% identical (Table 2).

The PUUV L segments were more than 81% identical at the nucleotide level, while the amino acid sequences of the L segment were at least 93% identical among all PUUVs (Table 3).

Phylogenetic analyses

We examined the evolutionary relationship between DTK/Ufa-97 and other hantaviruses by performing phylogenetic analyses of the S, M, and L genome segments using the neighbor-joining method. The DTK/Ufa-97 strain was determined to be a member of the PUUV group, and all three

segments of the DTK/Ufa-97 strain were determined to be members of the Russia/Bashkiria-Saratov lineage (Figs. 3A and 3B). The PUUV branching patterns were consistent with geographical origin, and PUUVs from European Russia (i.e., including the Bashkiria-Saratov, Tataria, and Samara PUUVs from the Volga river region) occupied a single cluster. The Russian/Omsk PUUVs clustered with the Sotkamo strain (Fig. 3A).

Table 1. Nucleotide and amino acid identities of the hantavirus S segment[a].

		ucleotide identities (%)									
		Puumala virus (PUUV)								HTNV[b]	SNV[c]
		Ufa-97[d,e]	CG1820	P360[e]	K27[e]	CG17	Fs808[e]	Kazan	Sot[f]	76-118	NM-H10
VUUP	Ufa-97	—	99.9	100	99.5	99.5	93.9	93.9	85.3	62.2	67.8
	CG1820	99.7	—	99.9	99.4	94.4	93.8	93.8	85.2	62.1	67.8
	P360	100	99.7	—	99.5	99.5	93.9	93.9	85.2	62.2	67.7
	K27	99.2	98.9	99.2	—	99.1	93.5	93.5	85.0	62.0	67.3
	CG17	100	99.7	100	99.2	—	94.2	94.4	85.5	62.7	67.5
	Fs808	99.4	99.2	99.4	98.6	99.4	—	94.8	84.6	62.6	67.8
	Kazan	98.9	98.6	98.9	98.0	98.9	98.9	—	84.5	62.5	68.1
	Sotkamo	96.9	96.6	96.9	96.3	96.9	96.9	96.8	—	63.3	66.7
	HTNV	59.3	59.0	59.3	58.4	59.3	59.6	59.3	59.6	—	61.3
	SNV	69.7	69.9	69.7	69.1	69.7	69.9	69.9	69.1	61.4	—
		Amino acid identities (%)									

[a] Nucleotide region to be compared : nt 172-1,239
[b] Hantaan virus
[c] Sin Nombre virus
[d] DTK/Ufa-97 strain
[e] PUUV originated from HFRS patients
[f] Sotkamo strain

Table 2. Nucleotide and amino acid identities of the hantavirus M segment[a].

		Nucleotide identities (%)									
		Puumala virus (PUUV)								HTNV[b]	SNV[c]
		Ufa-97[d,e]	CG1820	P360[e]	K27[e]	CG17	Kazan	Sot[f]	Umea[e]	76-118	NM-H10
VUUP	Ufa-97	—	99.6	99.8	99.8	99.4	85.8	82.8	80.3	60.0	66.7
	CG1820	99.3	—	99.5	99.5	99.1	85.7	82.8	80.3	60.1	66.7
	P360	99.8	92.3	—	99.9	99.3	86.0	83.1	80.5	60.2	66.9
	K27	99.7	99.1	99.7	—	99.4	86.3	83.2	80.8	60.5	67.3
	CG17	99.6	99.0	99.6	99.6	—	86.0	83.1	80.5	60.1	66.6
	Kazan	95.0	94.5	95.1	96.8	95.2	—	84.7	80.6	59.5	66.0
	Sotkamo	92.6	92.2	92.7	94.2	92.7	93.2	—	80.7	59.0	66.7
	Umea	88.9	88.4	88.9	91.1	89.1	89.4	89.3	—	59.8	65.9
	HTNV	53.4	51.9	52.2	54.0	53.3	54.5	53.6	51.8	—	58.9
	SNV	65.5	64.5	64.8	67.2	64.8	64.0	64.7	64.0	54	—
		Amino acid identities (%)									

[a] Nucleotide region to be compared: nt 53-3,494
[b] Hantaan virus
[c] Sin Nombre virus
[d] DTK/Ufa-97 strain
[e] PUUV originated from HFRS patients
[f] Sotkamo strain

Table 3. Nucleotide and amino acid identities of the hantavirus L segment[a].

		Nucleotide identities (%)							
		Puumala virus (PUUV)					HTNV[b]	SEOV[c]	SNV[d]
		Ufa-97[a,e]	CG1820	Kazan	Sot[g]	Umea[f]	76-118	80-39	NM-H10
VUUP	Ufa-97	—	99.8	87.1	84.5	81.3	67.0	67.1	71.0
	CG1820	99.8	—	87.1	84.6	81.4	67.0	67.2	71.0
	Kazan	99.1	98.9	—	85.0	81.9	67.1	66.9	71.7
	Sotkamo	97.4	97.2	97.4	—	82.1	66.8	67.8	71.3
	Umea	93.9	93.8	94.1	93.2	—	66.5	66.3	69.9
	HTNV	68.8	68.8	68.8	68.6	67.2	—	74.3	66.7
	SEOV	68.5	68.3	68.2	68.4	66.8	84.7	—	67.1
	SNV	77.8	77.1	77.4	77.6	76.1	68.9	68.9	—
		Amino acid identities (%)							

[a] Nucleotide region to be compared: nt 52-6,524
[b] Hantaan virus
[c] Seoul virus
[d] Sin Nombre virus
[e] DTK/Ufa-97 strain
[f] PUUV originated from HFRS patients
[g] Sotkamo strain

Table 4. Antigenic characteristic of Ufa-97 and other hantavirus strains.

MAbs against glycoprotein of HTNV	Antigenic site	IFA titer[a]					
		Puumala			Hantaan	Amur	Seoul
		Ufa-97[b]	Kazan	Sotkamo	76-118	H5	SR-11
8B6	Gn-a (1)	−	−	−	+++	+++	+
6D4	Gn-a (2)	−	−	−	−	−	−
10F11	Gn-a (2)	+	+	−	++	+++	+
2D5	Gn-b	−	−	−	++	++	+
3D5	Gn-b	−	−	−	+	+	+
16D2	Gn-b	−	−	−	++	++	−
HCO2	Gc-a (1)	−	−	−	++	++++	++++
16E6	Gc-a (2)	−	−	−	++	+++	++
EB06	Gc-b	+	+	+	++	+++	+
11E10	Gc-c	+++	+++	++	+++	+++	−
17G6	Gc-d	+	+	+	++	+++	+
5B7	Gc-d	++	+++	++	+++	+++	+++
20D3	Gc−e	+	+	−	++	+++	−
8E10	Gc-f(1)	+++	+++	+++	++	+++	++
1G8	Gc-f(1)	+++	+++	++	+++	+++	++
3B6	Gc-f(1)	+++	+++	+	+++	++	++
23G10-1	Gc-f(2)	−	−	−	++	++	++
7G6	Gc-f(2)	−	−	−	++	+++	++
18F5	Gc-f(2)	−	−	−	++	+++	+

[a] Antibody reactivity is defined as: −,<1:10; +,1:10; ++,1:100; +++,1:1,000; ++++,>1:1,000
[b] DTK/Ufa-97 strain

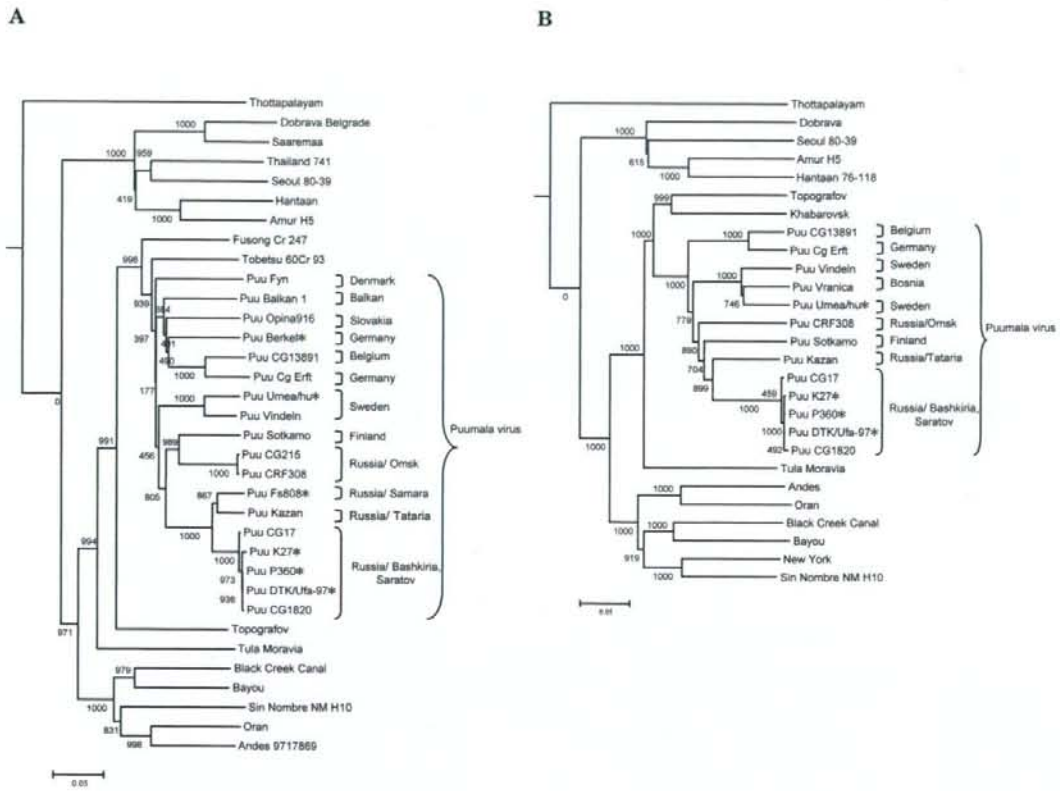

Fig. 3. Phylogenetic analysis of hantaviruses.
The nt sequences of the S, M, and L segments were obtained from the Genome Sequence Database. Multiple sequence alignment was performed using ClustalX software (ver. 2.0), and a phylogenetic tree was derived using the neighbor-joining method. Bootstrap values resulting from 1,000 replications are listed above each branch. A) Phylogenetic analysis of the 1068 nt S segment (i.e., approximately nucleotides 172 to 1239). The accession numbers for the S segment sequences are as follows: Thottapalayam, AY526097; Dobrava Belgrade, L41916; Saaremaa, AJ616854; Thailand 741, AB186420; Seoul 80-39, AY273791; Hantaan, M14626; Amur H5,AB127996; Fusong Cr 247, EF442087; Tobetsu 60Cr 93, AB010731; Puu Fyn, AJ238791; Puu Balkan1,AJ314600; Puu Opina 916, AF294652; Puu Berkel, L36943; Puu CG13891, U22423; Puu Cg-Erft, AJ238779; Puu Umea/hu, AY526219; Puu Vindeln/L20Cg/83, Z48586; Puu Sotkamo, X61035; Puu CG215, AF367066; Puu CRF308, AF367070; Puu F-s 808, AF411446; Puu Kazan, Z84204; Puu CG17/Baskiria-2001, AF442613; Puu K27, L08804; Puu P360, L11347; Puu DTK/Ufa-97, AB297665; Puu CG1820, M32750; Topografov, AJ011646; Tula Moravia/5302v/95, Z69991; Black Creek Canal, L39949; Bayou, L36929; Sin Nombre NM H10, L25784; Oran 22996, AF482715; and Andes Chile-9717869, AF291702. B) Phylogenetic tree of the 3442 nt viral M segment (i.e., approximately nucleotides 53 to 3494). The M segment accession numbers are as follows: Thottapalayam VRC-66412, EU001329; Dobrava-Belgrade DOBV/Ano-Poroia/Af19/1999, AJ410616; Seoul 80-39, S47716; Amur H5,AB127993; Hantaan 76-118, Y 00386; Topografov, AJ011647; Khabarovsk, AJ011648; Puu CG13891, U22418; Puu Cg-Erft, AJ238778; Puu Vindeln, Z49214; Puu Vranica, U14136; Puu Umea/hu, AY526218; Puu CRF308/Omsk, AF442617; Puu Sotkamo, X 61034; Puu Kazan, Z84205; Puu CG17/Baskiria-2001, AF442614; Puu K27, L08754; Puu P360, L08755; Puu DTK /Ufa97, AB297666; Puu CG1820, M29979; Tula Moravia/5302v/95, Z69993; Andes Chile-9717869, AF291703; Oran O122996, AF028024; Black Creek Canal, L39950; Bayou, L36930; New York NY-2,U36803; and Sin Nombre NM H10, L25783. Asterisks indicate viral strains of human origin.

Table 5. Cross-focus reduction neutralization titers of immune sera specific Ufa-97 and other representative hantaviruses.

Serum	Virus					
	Puumala			Hantaan	Amur	Seoul
	Ufa-97[a]	Kazan	Sotkamo	76-118	H5	SR-11
Ufa-97	**160**[b]	160	160	40	40	20
Kazan	160	**160**	320	80	80	20
Sotkamo	160	160	**640**	80	20	20
Hantaan	40	20	20	**160**	20	40
Amur	40	40	20	80	**160**	20
Seoul	20	<10	20	20	20	**40**

[a] DTK/Ufa-97 strain
[b] Neutralizing antibody titers to the homologous viruses are shaded and bold. Titers are shown as the reciprocal of the dilution that resulted in at least 80% reduction in focus, compared with the control (i.e., no antibody)

Antigenic characteristics of DTK/Ufa-97

The antigenic profile of the DTK/Ufa-97 strain was compared with that of other hantaviruses using IFA with a MAb panel. Six of the MAbs used in this study were specific to the HTNV envelope Gn glycoprotein, while 13 were specific to the Gc glycoprotein (Table 4). All MAbs specific to HTNV glycoproteins cross-reacted with AMRV, yielding very similar patterns. In addition, some MAbs specific to HTNV glycoproteins cross-reacted with SEOV, yielding somewhat dissimilar patterns (Table 4). Of the MAbs specific to antigenic sites of the Gn-a, Gn-b, Gn-a, and Gn-f(2) envelope glycoproteins, only MAb 10F11 cross-reacted with any of the PUUV strains. However, MAbs specific to the antigenic sites of the Gc-b, Gc-c, Gc-d, Gc-e, and Gc-f(1) glycoproteins reacted with all hantaviruses tested, including the PUUV strains (Table 4). In general, the PUUV strains exhibited similar reaction patterns to the latter group of MAbs (Table 4).

Cross-neutralization test

To further investigate the antigenic characteristics of DTK/Ufa-97, hamsters were infected with the DTK/Ufa-97, Kazan, and Sotkamo strains and immune sera were collected. These sera were then used to neutralize homologous or heterologous PUUV strains. All sera proved effective at high neutralizing titers (i.e., dilutions of 1:160 to 1:640) (Table 5). The PUUV immune sera exhibited lower neutralizing titers against HTNV, AMRV, and SEOV. However, immune sera specific to HTNV, AMRV, and SEOV exhibited high neutralizing antibody titers to homologous viruses but lower titers to heterologous viruses. These results indicate that the antigenic properties of DTK/Ufa-97 are similar to those of other PUUVs, with regards to the induction of neutralizing antibodies.

Discussion

Hemorrhagic fever with renal syndrome has the highest incidence and morbidity of all human zoonotic viral infections in Russia. Approximately 97% of HFRS cases are caused by PUUV in the European regions of Russia, whereas 3% of HFRS cases are caused by HTNV, SEOV and AMRV in the far-Eastern regions of the country[18,30]. An HFRS vaccine is urgently needed, as morbidity rates are high and approximately 12.5 million people (i.e., 25% of the population) in European regions are at risk for PUUV infection. Several hantavirus vaccines have been produced; however, none are effective against PUUV-induced HFRS. The difficulties in developing a PUUV vaccine stem from the low viral yield in cell culture. High titers of a potential vaccine strain, known as DTK/Ufa-97, can be cultured in SFM-grown Vero E6 cells. Here, we demonstrate that the DTK/Ufa-97 strain yields more viral RNA in infected Vero E6

cells and higher viral titers in the culture fluid of infected cells, when compared with the Sotkamo strain (Figs. 1 and 2). Our data clearly indicate that the DTK/Ufa-97 strain replicates more efficiently than the Sotkamo strain in SFM-cultured Vero E6 cells, as well as in MEM supplemented with FBS.

Minimum essential medium supplemented with FBS has been empirically shown to provide good conditions for cell growth and is commonly used in the formulation of growth media. However, FBS is a potential carrier of infectious agents such as fungi, bacteria, viruses, and prions, which could contaminate a final vaccine preparation[2]. Therefore, SFM is a safer alternative, as it does not contain components of animal or human origin. Several cell lines, including BHK-21[7] and Vero cells[17], have been successfully established in SFM.

A lot of PUUV sequences have been deposited in the DNA database, however, few full-length PUUV genome sequences are available, particularly for human isolates. Therefore, we determined the full-length nucleotide sequence of the DTK/Ufa-97 strain. To our knowledge, this is the first characterization of a Russian PUUV isolate from an HFRS patient. Our phylogenetic analysis revealed that the DTK/Ufa-97 strain is closely related to PUUV strains from the same geographic region (i.e., Bashkiria-Saratov) (Fig. 3). Our analysis identified four Russian PUUV clusters: Bashkiria-Saratov, Tataria, Samara, and Omsk (Fig. 3A). The first three clusters appeared on the same branch, while the Omsk cluster was more closely related to the Finland virus than to the other Russian viruses. This result is consistent with a previous report, in which the genetic identities of Russian and Finnish PUUV strains strongly correlated with their geographic origins[5,25].

The identities of predicted amino acid sequences in the S, M, and L segments were approximately 96.3%, 92.2%, and 93.2%, respectively among all PUUVs. However, the nucleotide sequences identities of the S, M, and L segments were much lower (i.e., approximately 84.5%, 80.3%, and 81.3%, respectively) (Tables 1-3). Few studies have examined the antigenic properties of PUUV; thus, we also examined the antigenic characteristics of DTK/Ufa-97 using MAbs. We compared the antigenicity of three PUUV strains (i.e., DTK/Ufa-97, Kazan, and Sotkamo), as well as other hantaviruses. The reaction patterns exhibited by the DTK/Ufa-97 strain were strikingly similar to those of other Russian and Finnish PUUVs (Table 4), but different than those of other hantaviruses, especially with regards to the Gn protein.

Envelope glycoproteins are presumed to play a major role in the induction of protective immunity and neutralizing antibodies[1,20]. Our cross-neutralization test demonstrated that a neutralizing antibody specific to the DTK/Ufa-97 strain also neutralized other PUUV strains at almost same antibody titer. In addition, antibodies to other PUUV strains cross-neutralized the DTK/Ufa-97 strain and homologous viruses (Table 5). Although the various PUUVs exhibit geographic-dependent genetic variation, they seem to share similar antigenic properties. Therefore, the DTK/Ufa-97 strain may prove useful in inducing protective immunity against a variety of PUUV strains, and may aid in the development of a DTK/Ufa-97-based vaccine.

Our findings revealed that the PUUV strain DTK/Ufa-97 grows well in Vero E6 cells cultured in SFM, and that it is antigenically similar to other PUUVs. These data may aid in the development of a PUUV vaccine strain based on DTK/Ufa-97.

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Epidemiology and Diagnosis of West Nile Virus Infection

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Abstract

West Nile (WN) virus produces a mosquito-borne infection manifesting in fever and, in serious cases, encephalitis. The virus that causes WN infections, WN virus, belongs to the family *Flaviviridae*, genus *Flavivirus*. The WN virus emerged in New York City in August, 1999, as the first case on the American continent, and seven human deaths were reported. Along with humans, deaths were observed among horses and various birds, such as crows. The WN virus epidemic expanded to the southern United States in 2000 and most of the rest of the country by 2004, extending as far as Argentina in 2007. Since there is a possibility of the WN virus spreading to Japan, where the Japanese encephalitis virus is also prevalent, the TaqMan assay was adapted to develop a sensitive, specific diagnostic test to differentiate WN virus from Japanese encephalitis virus.

Key words: epidemiology, diagnosis, West Nile virus infection

1. Epidemiology

1.1 Global epidemiology

The WN virus (WNV) was first isolated in 1937 from the blood of a febrile patient in the West Nile district of Uganda. WNV has since been found endemic over a wide range of areas in Africa, the Middle East, western Asia and Australia. Outbreaks of varying size occurred in Israel in 1941 and 1951-1954, and in Africa in 1974. After that, no large outbreaks were observed for 20 years, but from 1994 to 2000, WNV outbreaks occurred among humans and horses in Algeria in 1994, Morocco in 1996, Romania in 1996, Tunisia in 1997, the Czech Republic in 1997, the Congo in 1998, Italy in 1998, Israel in 1997-2000, Russia in 1999, France in 2000 and the United States in 1999-2002 (Marfin & Gubler, 2001).

1.2 Epidemiology on the American Continent

During the five years following the first diagnosis of WN encephalitis in August, 1999, in New York City, the WNV epidemic spread over 41 states. The total number of diagnosed patients was 26,997 and the total number of deaths was 1,008 (Table 1). (CDC (Centers for Disease Control and Prevention), 2008). Deaths in the United States were seen most often among persons older than 50 years and the average mortality rate was ca. 3.73%. Between 1999 and 2006, WNV was detected in 62 species of mosquitoes, with *Culex* species accounting for more than 98% of the total reported. Over the same period, 317 species of WNV-positive dead birds were reported, with American crows and blue jays accounting for more than 62% of the these cases. WNV outbreaks have also been reported among islands in the Caribbean Sea and in Argentina. The means of importation of the virus into the United States is unknown.

Table 1 Reported WNV disease cases in humans, United States, 1999-2007. (modified from CDC 2008)

Year	Total cases	Deaths
1999	62	7
2000	21	2
2001	66	9
2002	4,156	284
2003	9,862	264
2004	2,539	100
2005	3,000	119
2006	4,269	177
2007	3,022	76
Total	26,997	1,008

2. Causal Virus

WNV belongs to the family *Flaviviridae*, genus

Flavivirus and is included in the Japanese encephalitis (JE) virus serocomplex group. This group of viruses includes the St. Louis encephalitis virus of the United States and the Murray Valley encephalitis virus of Australia. The viral genome consists of single-stranded positive RNA and encodes three structural proteins (C, prM and E) and seven non-structural proteins.

WN virus isolates have been subjected to phylogenetic analysis, and can be subdivided into two major lineages. Lineage 1 includes most of the virus isolated since 1996, the American strains identified in 1999 and 2000, and the strains identified in Romania in 1996, Israel in 1999, and Volgograd, Russia in 1999. Lineage 2 includes most of the strains prevalent in Africa (Lanciotti *et al.*, 1999).

3. Transmission Cycle and Infection Route

The major transmission cycle of WNV occurs between mosquitoes and birds. The model of virus transmission proposed for the United States is shown in Fig. 1. Birds are the virus-amplifying hosts, and mosquitoes of the *Culex* species are the major vectors in many outbreaks. Humans and horses are infected by bites from virus-carrying mosquitoes, and while they show clinical symptoms such as fever and encephalitis, they are called 'dead-end hosts' as they do not serve as an infection source for mosquitoes, because the viremia in humans and horses is not high enough to infect the mosquitoes. Major vector species of mosquitoes in the United States are *Cx. pipiens, Cx. quinquefasciatus, Cx. restuans, Cx. salinarius* and *Cx. tarsalis*. Crows are often used as sentinel animals to monitor virus activity in the United States.

Routes of human infection other than mosquito bites include fetal infection through the placenta, infantile infection via breast feeding, infection due to blood transfusion or organ transplantation and laboratory-acquired infection during handling of the live virus or infected materials.

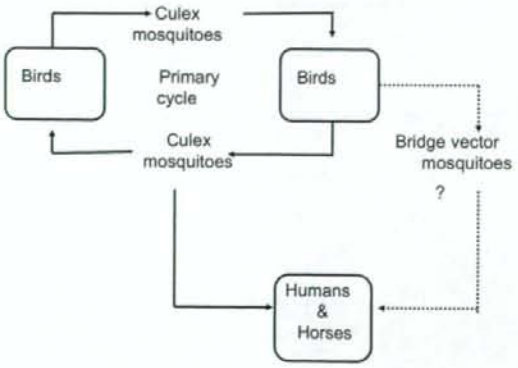

Fig. 1 Transmission cycle of WN virus. (modified from Campbell *et al.* 2002)

4. Clinical Symptoms

In humans, the incubation period of WNV is three to fifteen days following infection. Most cases are asymptomatic, but in some cases, fever and fatal encephalitis may develop. Mild cases show a febrile period of three to six days, headache, backache, myalgia, fatigue, rash and lymphadenopathy. Severe cases develop acute myelitis and encephalitis. The mortality rate of the outbreaks recorded in the United States has been approximately 3.73%.

In horses, the incubation period is five to ten days. Most infected horses are asymptomatic, but some show clinical symptoms. In the United States, reported symptoms include ataxia, weak legs, lying down, astasia, myospasm, fever, facial neuralgia, lip palsy, facial spasm, gnashing and blindness. The mortality rate, including euthanised cases, is 38%.

In birds, infection is asymptomatic, except in the United States and Israel. In the cases recorded in the United States, high mortality was observed among American crows and blue jays. Symptoms included depression, anorexia, weakness and loss of body weight. Severe cases manifest neurological signs such as ataxia, tremor, revolution and paresis. The clinical signs last for seven days.

5. Diagnosis

Diagnosis of WNV involves virus detection and serological examination. Virus isolation or virus genome detection is performed using sera or cerebrospinal fluids. IgM-captured ELISA, neutralization test, hemagglutination inhibition test and complement fixation test are used to detect WNV-specific antibodies. Acute phase sera, plasma, cerebrospinal fluids or post-mortem brain materials can all be used for virus isolation. For serological examination, paired sera from patients in the acute phase and convalescent phase should be collected and tested. Differential diagnosis should be considered for other flavivirus infections including JE, St. Louis encephalitis, tick-borne encephalitis, Murray Valley encephalitis and dengue. Other viral infections, such as alphavirus infection and herpesvirus infection, should be also considered.

5.1 Development of a sensitive diagnostic test for differentiation of WNV and Japanese encephalitis (JE) virus

Like WNV, JE virus is a mosquito-borne flavivirus from the JE virus (JEV) serocomplex, and causes encephalitis in humans and horses; it is widespread throughout most of Asia (Igarashi, 1992; Rosen, 1986). In areas where JEV is endemic, such as Japan, distinguishing between WNV and JEV is critical for correct identification of a WNV invasion. However, JE serocomplex flaviviruses cross-react antigenically and are thus not readily differentiated by serological methods (Martin, *et al.*, 2000). Molecular diagnostic methods

are therefore preferred, and the reverse transcriptase polymerase chain reaction (RT-PCR) has been used to develop sensitive and specific assays for the identification of WNV (Igarashi, *et al.*, 1994; Porter, *et al.*, 1993). Recently, more sensitive assays, such as fluorogenic real-time (TaqMan) PCR, SYBR Green-based real-time PCR and loop-mediated isothermal amplification (LAMP), have been developed for diagnostic detection of the WNV genome (Lanciotti, *et al.*, 2000; Papin, *et al.*, 2004; Parida *et al.*, 2004). In areas where JEV is endemic, it is necessary to perform specific assays for both WNV and JEV to make a definitive diagnosis. However, the diagnostic methods described above were designed to detect only WNV or, more specifically, the strain of WNV isolated in the United States. Here, we describe the development of a sensitive molecular diagnostic method that can detect and distinguish between WNV and JEV, using TaqMan RT-PCR analysis with a probe common to WNV and JEV strains (Sirato *et al.*, 2005).

To determine the sensitivity of the TaqMan assay, the assay was performed with serially-diluted cDNA from the NY99-6922 strain of WNV (Fig. 2). It was established that a cut-off (Ct) value of <40 and ΔRn signal of >0.5 indicated that WNV was present. The specificity of the primer sets was tested using various flaviviruses (Table 2). The TaqMan assay was performed using the indicated volume of cDNA synthesized from total RNA. The results indicated that the primer set for WNV could detect only WNV strains, including both lineage 1 and 2 viruses. The primer set for JEV could detect only JEV strains, including genotypes 1 (Ishikawa) and 3 (JaGAr01, Nakayama, and Beijing). The MVE virus, which is one of the JE serocomplex viruses, was not detected by either primer set. Flaviviruses of other serocomplexes, such as TBE virus, Dengue virus, Langat virus, and Powassan virus, were also not detected by either primer set.

To measure the sensitivity of the TaqMan assay for other WNV and JEV strains, the assay was performed

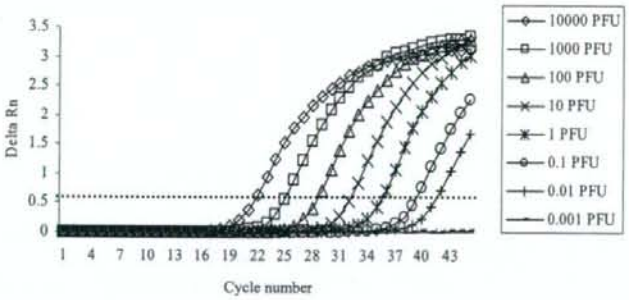

Fig. 2 Determination of the cut-off values for the TaqMan assay. The assay was performed with serially diluted cDNA from the NY99-6922 strain of WNV. The dotted line is drawn at ΔRn = 0.5. (Data from Shirato *et al.* 2005.)

Table 2 Specificity of the multi-probe TaqMan assay. (modified from Shirato *et al.* 2005)

		Primer set used			
		WNV primers		JEV primers	
Virus	Amount of template	Ct*	Result**	Ct	Result
WNV					
Lineage 1					
NY99-6922	1 μg of total RNA	16.8 ± 0.18	Pos	> 40.0	Neg
NY99-A301	1 μg of total RNA	17.7 ± 0.15	Pos	> 40.0	Neg
BC787	1 μg of total RNA	17.6 ± 0.03	Pos	> 40.0	Neg
6-LP	1 PFU	33.6 ± 0.00	Pos	> 40.0	Neg
6-SP	1 PFU	31.2 ± 0.07	Pos	> 40.0	Neg
Eg101	1 μg of total RNA	17.0 ± 0.04	Pos	> 40.0	Neg
Kunjin (OP393)	100 PFU	34.7 ± 0.20	Pos	> 40.0	Neg
Lineage 2					
FCG	1 μg of total RNA	17.2 ± 0.03	Pos	> 40.0	Neg
JEV					
Genotype 1					
Ishikawa	1,000 PFU	> 40.0	Neg	23.5 ± 0.03	Pos
Genotype 3					
Nakayama	1 μg of total RNA	> 40.0	Neg	20.1 ± 0.19	Pos
Beijing	1 μg of total RNA	> 40.0	Neg	15.8 ± 0.2	Pos
JaGAr01	1 μg of total RNA	> 40.0	Neg	16.3 ± 0.11	Pos

Pos=positive; Neg=negative
* Data are represented as the mean ± standard deviation.
** Ct values < 40 with ΔRn signals of > 0.5 were considered positive.

using serially diluted cDNA samples from titrated virus stocks (Table 3). At least 10^{-1} plaque-forming units (PFU) of virus was required for fluorescence detection by the TaqMan assay with WNV or JEV primer sets. However, the sensitivity for detection of Kunjin virus was lower than that of other viruses; at least 10 PFU of Kunjin virus were required for detection. Although the TaqMan assay was first performed using a 50 µl reaction volume, there was no change in sensitivity when the assay was performed using 25 µl or 12.5 µl reaction volumes. The TaqMan assay could detect 10^{-1} PFU or more of virus even in these smaller reaction volumes (data not shown). Therefore, all TaqMan assays were performed using a 25 µl reaction volume thereafter.

We also examined whether the primer sets used in this study could detect viruses in animal tissues. As Japan is a WNV-free country, we were unable to obtain archived clinical samples from animals naturally infected with WNV. Therefore, the TaqMan assay was performed using tissues from experimentally-infected mice. BALB/c mice were infected with 10^{6} PFU of the NY99-6922 strain of WNV via the intraperitoneal route, and tissue samples were collected on the indicated days post-infection (p.i.). Total RNA was extracted, and cDNA was synthesized and tested using the TaqMan assay. The assay detected viral RNA in blood, spleen and brain samples (Table 4). Viruses were detected at both the early (three days p.i.) and late (eight days p.i.) stages of infection. Plaque assays were also performed to detect viruses in infected animal tissues.

This paper describes a highly sensitive genetic diagnostic method that can detect WNV and JEV using TaqMan RT-PCR analysis with a single probe that is common to both WNV and JEV strains. WNV strains of both lineage 1 and 2 were successfully detected when using the primer set for WNV, and genotype 1 and 3 JEV were detected when using the primer set for JEV. Viral RNA was detected in experimentally-infected animal tissues. Therefore, it is considered likely that the method described here is sufficiently sensitive and specific for detecting WNV and JEV strains in both human and animal samples.

Table 3 Sensitivity of the multi-probe the TaqMan assay. (modified from Shirato *et al.* 2005)

	Virus dose (PFU)					
Virus	10^{3}	10^{2}	10^{1}	10^{0}	10^{-1}	10^{-2}
WNV						
Lineage 1						
NY99-6922	Pos*	Pos	Pos	Pos	Pos	Neg
6-LP	Pos	Pos	Pos	Pos	Pos	Neg
6-SP	Pos	Pos	Pos	Pos	Pos	Neg
Eg101	Pos	Pos	Pos	Pos	Pos	Neg
Kunjin (OP393)	Pos	Pos	Pos	Neg	Neg	Neg
Lineage 2						
FCG	Pos	Pos	Pos	Pos	Pos	Pos
JEV						
Genotype 1						
Ishikawa	Pos	Pos	Pos	Pos	Pos	Neg
Genotype 3						
JaGAr01	Pos	Pos	Pos	Pos	Pos	Neg

Pos=positive; Neg=negative
Ct values < 40 with ΔRn signals of > 0.5 were considered positive.

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Table 4 Detection of WNV and JEV in experimentally infected mouse tissues using the multi-probe TaqMan assay. (modified from Shirato, 2005)

						Primer set			
						WNV		JEV	
Infecting virus	Number	Days p. i.	Clinical signs	Tissue	Virus titer*	Ct	Result**	Ct	Result
NY99-6922	1	3	No signs	Blood	1.3	37.5	Pos	> 40.0	Neg
				Spleen	3.6	30.2	Pos	> 40.0	Neg
	2	3	No signs	Blood	1.0	39.9	Pos	> 40.0	Neg
				Spleen	3.8	30.6	Pos	> 40.0	Neg
	3	3	No signs	Blood	1.0	37.1	Pos	> 40.0	Neg
				Spleen	3.6	31.1	Pos	> 40.0	Neg
	4	8	Dead	Spleen	2.3	32.0	Pos	> 40.0	Neg
				Brain	> 7.0	14.4	Pos	> 40.0	Neg
	5	8	Moderate encephalitis	Blood	1.3	38.3	Pos	> 40.0	Neg
				Spleen	2.8	30.2	Pos	> 40.0	Neg
				Brain	5.6	24.5	Pos	> 40.0	Neg

Pos=positive; Neg, negative; n.d.=not detected
* The viral titer in blood samples is expressed as log PFU/ml and that of tissue samples is expressed as log PFU/g. The detection limits were 10 PFU/ml (blood) and 100 PFU/g (spleen and brain).
** Ct values < 40 with ΔRn signals of > 0.5 were considered positive.

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Seroepidemiological study on hantavirus infections in India

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Summary Hantaviruses are etiological agents of hemorrhagic fever with renal syndrome in many parts of Asia and Europe. There has been no documented case of hantavirus disease from India, although serological evidence exists. We investigated the prevalence of hantavirus in the Indian population and tried to identify potential risk groups for hantavirus infections. The presence of hantavirus-specific IgG antibodies was prospectively evaluated in 661 subjects belonging to different groups, i.e. patients with chronic renal disease, warehouse workers and tribal members engaged in rodent trapping. Healthy volunteer blood donors were included as a control group. Thirty-eight seropositive samples were found using a combination of a commercial ELISA followed by an indirect immunofluorescence assay. Western blot using recombinant Hantaan virus nucleocapsid antigen confirmed the presence of anti-hantavirus IgG in 28 (74%) of the 38 sera tested. This study confirms the presence of hantaviruses in India and warrants increasing awareness of the problems of emerging pathogens and the threats they may pose to the public health system.

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1. Introduction

Hantaviruses represent a group of emerging viruses. The genus *Hantavirus*, belonging to the family Bunyaviridae, comprises more than 20 species that can cause two diseases in humans: hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia and hantavirus cardiopulmonary syndrome (HCPS) in the Americas (Lednicky, 2003). Almost all hantaviruses are maintained in rodents of the family Muridae, and are transmitted to humans via aerosolized urine, saliva and feces of infected rodents.

Although hantavirus infections were recognized in Asia for centuries, it was only during investigations initiated after the Korean conflict in the 1950s, during which thousands of UN soldiers were affected by HFRS, that the Hantaan virus (HTNV) serotype was isolated. The circulation of hantavirus serotypes, namely Seoul virus (SEOV) and Thailand virus (THAIV), has been demonstrated in several Southeast Asian countries, i.e. Thailand, Cambodia, Viet Nam and Indonesia (Plyusnina et al., 2004; Reynes et al., 2003; Truong et al., 2004). Recently a report from Thailand documented the first serological evidence of THAIV causing HFRS in humans (Pattamadilok et al., 2006). The Thottapalyam virus (TPMV), which was isolated from an insectivore, *Suncus murinus*, in 1964, is the only known hantavirus species indigenous to India (Cary et al., 1971).

The association of chronic renal disease and hantavirus seropositivity has frequently been speculated upon as studies conducted in the United States (Baltimore) suggested that hantavirus seropositives had higher rates of chronic renal disease and hypertensive renal disease than age-matched seronegative controls (Glass et al., 1990). Similar studies have been reported from Israel (George et al., 1998) and Egypt (Botros et al., 2004). An epidemiological study from Taiwan has reported detection of anti-hantavirus antibody in various risk groups such as garbage collectors and animal handlers (Chen et al., 1998).

Although the isolation of TPMV pre-dates that of HTNV, interest in hantaviruses was revived in India in 2005, with two reports on the serological evidence of hantavirus infections in patients with febrile illnesses (Chandy et al., 2005; Clement et al., 2006). However, seroepidemiological surveys have not been reported from India, and this study is the first attempt to investigate the epidemiology of hantavirus infections in India.

2. Materials and methods

2.1. Study population

The study subjects belonged to different groups and were recruited at the Christian Medical College, Vellore, south India. Healthy volunteer blood donors ($n = 360$) comprised the control group. The potential risk groups included 99 sera from asymptomatic Irulas, a tribal community living in Tamil Nadu, a state in south India (the tribe members are professional rat catchers and eat rats) and 51 sera from people working in warehouses (warehouse workers) in and around Vellore. An additional group was represented by 151 serum samples from patients with chronic renal disease with serum creatinine and urea levels of >1.4 mg/dl and >40 mg/dl, respectively.

The mean (±SD) age of the subjects in the Irula group was 32 (±11.2) years, and this group included 41 males and 58 females; in the warehouse workers it was 38 (±10.17) years and all were males; and in the renal disease patients it was 43 (±12.11) years and there were 47 females and 104 males. The subjects in the control group were aged 18–60 years.

The sample size was calculated based on a previous study of hantavirus infections in India (Chandy et al., 2005). The study period was from August 2004 to May 2007. Samples from healthy blood donors and patients with chronic renal disease were collected at the Department of Clinical Virology, Christian Medical College, Vellore and were included in the study by convenient sampling. All samples collected from asymptomatic Irulas and warehouse workers were included in the study. Written informed consent was obtained before collecting blood.

2.2. ELISA and immunofluorescence assay

Serological screening was done using a commercial (ISO certified) hantavirus IgG ELISA (Focus Technologies, Cypress, CA, USA) according to the manufacturer's instructions. This ELISA uses a cocktail of hantavirus antigens of HTNV, SEOV, Puumala virus (PUUV), Sin Nombre virus (SNV) and Dobrava-Belgrade virus (DOBV) to coat the polystyrene microwells and can detect IgG antibodies against these serotypes. The screening ELISA is an indirect test in which the optical density (OD) is directly proportional to the antigen-specific IgG antibodies present in the sample. The results were obtained by comparison of the sample OD readings with reference cut-off OD readings.

Results were reported as index values relative to the cut-off calibrator. To calculate index values, each sample OD value was divided by the mean of the cut-off calibrator OD values.

Sera positive by ELISA were re-tested by an indirect immunofluorescence assay (IFA) using HTNV-infected Vero E6 cells as antigens (Yoshimatsu et al., 1993). The secondary antibody used was fluorescein-isothiocyanate-conjugated rabbit anti-human IgG (DakoCytomation, Glostrup, Denmark). For the IFA, all spots with at least half of the infected cells showing a characteristic apple green granular cytoplasmic fluorescence with $\geq 2+$ intensity were scored positive.

Finally, a sample was considered positive if reactive by both ELISA and IFA. The screening ELISA and IFA were evaluated using a panel of positive and negative control sera kindly supplied by the European Network for Diagnostics of Imported Viral Diseases [ENIVD (Biel et al., 2003)].

2.3. Western blot analysis

Western blot analysis was performed on 38 positive sera using recombinant nucleocapsid protein (NP) of HTNV (Fojnica strain) as antigen (Razanskiene et al., 2004). Sera at dilutions of 1:1000 were applied to the membrane, and goat anti-human IgG conjugated with alkaline phosphatase (Genelabs Diagnostics Pte Ltd, Singapore Science Park, Singapore 118259, Republic of Singapore) at 1:1000

Table 1 Results of evaluation of the screening ELISA and Hantaan virus-immunofluorescence assay (IFA) using European Network for Diagnostics of Imported Viral Diseases (ENIVD) sera

ENIVD no.	Sample type	IgG level	Origin	Hantavirus strain	Serum status	ELISA result	IFA result
2	Positive serum	++	Sweden	Puumala	Convalescent	+	+
8	Positive serum	+	Kosovo	Dobrava	Convalescent	+	+
9	Positive serum	++	Sweden	Puumala	Convalescent	+	+
16	Positive serum	++	Sweden	Puumala	Convalescent	+	+
17	Positive serum	+	Finland	Puumala	Convalescent	−	−
4	Negative serum	−	Germany	−	Control	−	−
12	Negative plasma	−	Germany	−	Control	−	−
18	Negative serum	−	Germany	−	Control	−	−
19	Negative serum	−	Germany	−	Control	−	−

+, positive; ++, strong positive; −, negative.

dilutions was used as the secondary antibody. Thereafter the membrane was developed with substrate, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT). In the Western blot the molecular size of the expected product was about 49 kDa.

2.4. Statistical analysis

The statistical analysis was done using EpiInfo version 6.04b (CDC, Atlanta, GA, USA) to compare two categorical variables. Percentages were calculated for categorical outcomes (positives/negatives). A P-value <0.05 was considered significant.

3. Results

ENIVD sera were used to evaluate the commercial IgG ELISA and the IFA using HTNV antigen; these assays could detect anti-Puumala virus and anti-Dobrava virus IgG-positive sera. However, one ENIVD anti-Puumala virus serum (origin Finland) could not be detected by either of these assays (Table 1).

In the initial screening, 661 serum samples were tested by a commercial IgG ELISA (Table 2). Forty-seven of the 661 sera were found to be reactive in the ELISA. The majority (38/47) of the ELISA-reactive sera were also detected by IFA using HTNV antigen. Seropositivity in the Irula tribal group (11%) was significantly higher than in the control group (4%, $P<0.05$). There was no statistically significant difference between seropositivity in the chronic renal disease patient group (7%) compared to that of the control group. The level of seropositivity in the warehouse workers (2%) was very similar to that of the control group. Sex as a demographic factor was not significantly associated with hantavirus infections in the different subject groups, suggesting that males and females are equally likely to contract hantavirus infections. The mean age of the seropositives in the renal disease patient group, the Irulas and the warehouse workers was 50 (SD ± 11.29), 29 (SD ± 8.9) and 31 years, respectively. Twenty-eight of the 38 positive sera (74%) were positive by Western blot (Table 2).

4. Discussion

Studies on hantavirus infections in India are in the early stages. The data given here represent the first attempt to characterize the epidemiology of hantavirus infections in India and strengthen previous reports on serological evidence of hantavirus infections in India (Chandy et al., 2005; Clement et al., 2006).

Forty-seven of 661 serum samples were positive by ELISA. Serology is the mainstay of diagnosis of hantavirus infections. ELISAs are highly sensitive and are the preferred diagnostic tool for serological surveys. The commercial ELISA used in this study uses a cocktail of six antigens, and can be used in areas where the circulating hantavirus species are unknown. The specificity of the hantavirus assays used in the study is acceptably good, as evaluated by the ENIVD-negative control sera. There may be problems with diagnostic sensitivity, as one ENIVD anti-Puumala IgG-positive serum was not detected by both the assays.

Table 2 Results of serological studies

Group tested	No. tested	No. positive by ELISA (%)	No. positive (ELISA and HTNV-IFA) (%)	No. positive/no. tested by Western blot (%)
Blood donors	360	19 (5)	16 (4)	11/16 (69)
Renal disease patient group	151	14 (9)	10 (7)	9/10 (90)
Irulas	99	12 (12)	11 (11)	7/11 (64)
Warehouse workers	51	2 (4)	1 (2)	1/1 (100)
Total	661	47 (7)	38 (6)	28/38 (74)

HTNV-IFA: Hantaan virus-immunofluorescence assay.

The lower seroprevalence observed in the IFA and Western blot analysis might be due to the HTNV antigen, which suggests that hantavirus species other than HTNV are circulating and causing human disease in India. Alternatively, we cannot exclude the possibility that the ELISA picked up false positives.

It has been documented that TPMV is phylogenetically and antigenically quite distinct from the other well-characterized hantaviruses (Song et al., 2007), and although antibodies against hantavirus NP are cross-reactive between different hantavirus species, we cannot speculate about the efficiency of the assays used in the study to detect antibodies against TPMV. Moreover, there may be other hantavirus species circulating in India that may be as diverse as TPMV, and in the case of an antigenic mismatch the assays used here may fail to detect seropositives. It is thus important to define the hantavirus species circulating in India and develop sensitive assays using homogeneous antigens.

In this study, the renal disease patient group appears to have a higher risk of hantavirus seropositivity compared with the control group, but the difference is not statistically significant. It is still not clear whether patients with chronic renal disease are at a higher risk of acquiring hantavirus infections or that hantavirus infections by themselves contribute to the development of chronic renal disease. These results are preliminary, and follow-up studies are needed to prove any significant association of hantavirus infections with chronic renal disease.

The tribal group in this study has a relatively high level of contact with rodents, as they are traditionally rat catchers and also eat rats. They display a high seropositivity when compared with the control group. By contrast, warehouse workers showed a low seropositivity in our study (2%). This may reflect a lower risk of contracting hantavirus infections due to the fact that many warehouses in India adopt stringent rodent-control measures.

No well-documented hantavirus case, as defined by virus isolation or molecular evidence, has been reported from India to date. However, our study confirms that one or more hantaviruses are circulating in the Indian population and indicates that the threat from emerging pathogens must be continually assessed. Studies to identify the hantaviruses that might cause problems for public health systems are important, as they will aid the development of new strategies for the prevention and control of such emerging infections.

Authors' contributions: GS and PA designed the study protocol; SC carried out the immunoassays and drafted the manuscript; KY, RGU, MM, MO and JA supplied IFA slides and Western blot strips and helped analyse results obtained; RP, GTJ, VB, JM and JM helped with the identification of subjects in the various groups and collection of samples. All authors read and approved the final manuscript. GS is guarantor of the paper.

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Conflicts of interest: None declared.

Ethical approval: The institutional research ethics committee of the Christian Medical College, Vellore, Tamil Nadu, India (R.C. Min. No. 5838 dated 21 February 2006) and the Indian Council for Medical Research (ICMR), New Delhi, India.

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