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Molecular Epidemiological and Serological Studies of Hantavirus Infection in Northern Vietnam

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ABSTRACT. The distribution of anti-hantavirus antibodies in humans and rodents in northern Vietnam was examined. In total, 837 serum samples from healthy humans (617) and patients with fever (220), living in six different areas were screened for IgG antibodies against Hantaan or Seoul virus (SEOV) by ELISA, IFA, and Western blot analysis. Antibody-positive sera were identified in 7/617 (1.1%) healthy donors, 5/150 port workers in the port of Hai Phong, and 2/185 residents of Ha Nam Province. In comparison, positive sera were detected in 5/220 (2.3%) fever patients in the provinces of Ha Nam (1/58) and Thanh Hoa (4/146). Antibody-positive *Rattus norvegicus* were found in the provinces of Ha Nam (7/52) and Thanh Hoa (1/67), in Haibatrung District (7/43) in Hanoi, and in Hai Phong Port (21/62), while antibody-positive *R. rattus* (2/17) were found in Hai Phong Port. Part of the Gc region from the viral genome was amplified by RT-PCR using lung tissue samples from *R. norvegicus* in Haibatrung (2/7) and Hai Phong Port (7/9), but not from *R. rattus* (0/2). Viral sequences were located in the SEOV clade and formed a single lineage with Indonesian SEOV, suggesting that Vietnamese SEOV is part of a distinct lineage among Asian SEOVs.
KEY WORDS: HFRS, *Rattus*, rodent, Seoul virus, zoonosis.

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Hantaviruses are enveloped RNA viruses that belong to the *Hantavirus* genus in the *Bunyaviridae* family. The hantavirus virion contains three negative-sense RNAs, designated S, M, and L, based on their relative sizes; they encode a nucleocapsid protein (N), enveloped glycoproteins (Gn and Gc), and an RNA-dependent RNA polymerase (L protein), respectively [8]).

Hantaviruses are found primarily in rodents and are the causative agents of two severe viral zoonoses, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). A close relationship exists between each viral species and its particular rodent reservoir species; thus, endemic areas of HFRS and HPS are confined to areas inhabited by the reservoir rodents. Consequently, HFRS exists throughout Eurasia, while HPS is found in North and South America [23].

The hantaviral species that have been causally associated with HFRS, Hantaan virus (HTNV), Seoul virus (SEOV), and Dobrava/Belgrade virus (DOBV), are carried by rodents in the subfamily Murinae (Old World rats and mice), while Puumala virus (PUUV) is carried by rodents in the subfamily Arvicolinae (voles and lemmings) of the family Muridae [20].

The total number of HFRS cases per year is about 60,000–150,000, but more than 90% of these cases occur in East Asian countries, including China, Russia, and Korea [14]. HFRS is caused by HTNV, which is carried by striped field mice (*Apodemus agrarius*), SEOV, which is carried by brown rats (*Rattus norvegicus*), and PUUV, which is carried by voles (*Myodes* spp.) [23]. Epidemiological and epizootiological studies have shown that hantavirus infections spread in both humans and rodents in South and Southeast Asia [6, 9, 22]. Because most of the affected animals are *Rattus* spp., SEOV infections introduced by infected brown rats through international freight transportation is suspected. However, the existence of different hantaviruses has also been reported. For example, Thailand virus (THAIV) is the only hantavirus species carried by *Bandicota indica*, of the subfamily Murinae, in Thailand [9], while Thottapalayam virus (TPMV), which was isolated from insectivore mammals in India [5], is antigenically and genetically quite distant from other rodent-derived hantaviruses. Several studies have reported antibody-positive human sera against THAIV [19, 27] and TPMV [18] in Thailand. Further, Cao Bang virus was isolated from a Chinese mole shrew (*Anourosorex squamipes*) captured in Cao Bang Province in Vietnam [26]. These data indicate that various hantavirus species are circulating throughout South and Southeast Asia. Because the clinical symptoms of leptospilosis and other febrile illnesses are similar to those of HFRS, undiagnosed cases of HFRS may exist in patients with febrile illnesses of unknown origin (FUO). However, limited data are available regarding human and rodent infections with hantaviruses in South and Southeast Asia.

In this study, human and animal sera derived from various districts of Vietnam were screened for evidence of hantavirus infection. Additionally, hantavirus genomes were amplified from lung tissue collected from rodents captured

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in hantavirus-positive areas. Finally, the relationship between Vietnamese hantaviruses and other hantaviruses found in Asia was analyzed phylogenetically.

MATERIALS AND METHODS

Viral strains and cells: HTNV strain 76–118 [15], SEOV strain SR-11 [11], THAIV strain Thai749 [9], TPMV strain VRC-66412 [5], and PUUV strain Sotkamo [4] were used as representative strains of HTNV, SEOV, THAIV, TPMV, and PUUV respectively. All of the viruses were propagated in Vero cells (clone E6; ATCC C1008) prior to their molecular and antigenic characterization or use in a focus reduction neutralization test (FRNT).

Human sera and methods for antibody detection: In total, 837 serum samples were obtained from healthy individuals or patients. The sera were identified by numbers to prevent subject identification. The use of the sera for the investigation of fever of unknown origin (FUO) in Vietnam was explained to the blood donors orally. All blood donors provided informed consent. The FUO were initially suspected to be dengue fever; however, they were seronegative for dengue virus. Sera were collected in the Vietnamese provinces of Bac Giang, Hai Phong, Ha Nam, Ha Noi, Hoa Binh, and Than Hoa (Table 1, Fig. 1). Screening for anti-hantavirus IgG was performed by ELISA at a serum dilution of 1:200. Recombinant N proteins of HTNV, PUUV, and TPMV were expressed by a recombinant baculovirus system and designated rNP-HTNV, rNP-PUUV and rNP-TPMV, respectively. The three different rNPs were used as ELISA antigens, as described previously [18, 19]. Baculovirus-expressed bornavirus P24 antigen was used as a negative control. All positive sera were subjected to Western blotting using recombinant SEOV antigen and indirect immunofluorescent antibody (IFA) testing, using SEOV-infected Vero E6 cell antigen, as previously described [31]. Those sera confirmed as positive by ELISA, Western blotting, and IFA were subjected to serotyping ELISAs to determine the type of hantavirus [3, 17]. Briefly, the serum samples were diluted 1:200 and then applied to microtiter plate wells coated with truncated N antigen lacking 49 amino acids from the N-terminal end. The truncated N antigens used for serotyping ELISA were prepared from HTNV, SEOV, and THAIV by a recombinant baculovirus system and were designated HTNV50, SEOV50, and THAIV50, respectively. Screening for virus-reactive IgM was performed by μ-capture ELISA using recombinant N protein from HTNV, as described previously [16]. Sera from four HFRS patients who had been previously diagnosed with HTNV, SEOV, THAIV, and PUUV, by FRNT, were used as positive controls, while negative human control sera (NHS), which were confirmed to contain no antibodies against hantaviruses, were used as negative controls.

Animal sera and antibody detection: In total, 442 serum samples were obtained from Rattus spp. or shrews (*Suncus murinus*) captured at six locations in Vietnam between 1998 and 2006 (Table 2, Fig. 1). The sera were screened by indirect IgG ELISA using *Escherichia coli*-expressed His- and NUS-tagged partial N protein (103 amino acids from the N-terminus) from HTNV strain 76–118 or full-length N protein from TPMV, expressed using a pET43.1 vector system (Novagen) [18]. The sera were then diluted 1:200 and screened by ELISA. The presence of bound antibodies was detected using a horseradish peroxidase-conjugated goat

Table 1. Results of serological screening for hantaviruses in human sera

Location	Healthy		Patient	
	Tested	Positive	Tested	Positive
Bac Giang	20	0	–	–
Hai Phong*	150	5	16	0
Ha Nam	185	2	58	1
Ha Noi	73	0	–	–
Hoa Binh	14	0	–	–
Thanh Hoa	175	0	146	4
Total (%)	617	7 (1.1)	220	5 (2.3)

* Healthy port workers of Hai Phong port belonged to the "healthy" group of Hai Phong province. The patient group of Hai Phong province did not contain patients from Hai Phong port workers. Numbers in parentheses indicate the percentage positive.

Fig. 1. Map showing the collection sites for the human serum samples and trapping sites for the rodents in Vietnam (•).

Table 2. Trapping sites, collected rodent species, and seropositivity for hantavirus

Location	Species	No. Positive (PCR positive/tested)	No. Tested (% positive)
Ha Nam	*Rattus norvegicus*	7	52 (13.5)
Thanh Hoa	*R. norvegicus*	1	67 (1.5)
Tay Nguyen	*R. norvegicus*	0	38
	R. exulans	0	86
	Suncus murinus	0	22
	R. hosaensis	0	5
	R. rattus	0	1
Vin Phuc	*R. norvegicus*	0	2
	R. rattus	0	21
Ha Noi Haibatrung District	*R. norvegicus*	7 (2/7)	43 (16.3)
	R. rattus	0	7
	S. murinus	0	19
Hai Phong Port	*R. norvegicus*	21 (7/9)	62 (33.9)
	R. rattus	2 (0/2)	17 (11.8)
Total		38	442

anti-rat IgG antibody (Zymed Laboratories Inc.) or Protein A (Prozyme). The antibody-positive sera were then subjected to Western blotting using recombinant hantavirus N proteins from HTNV strain 76–118 and baculovirus-expressed TPMV (strain VRC-66412), as described previously [18, 32], and IFA using HTNV strain -76–118- and TPMV strain VRC-66412-infected Vero E6 cells as antigens. As positive controls, three serum samples from Wistar rats that had been experimentally infected with SEOV strain SR-11 were used. As negative controls, sera from five wild-trapped, uninfected rats from Japan were used [29]. For the shrew sera, experimentally infected and uninfected shrew serum samples were used as positive and negative controls [18].

IFA assay: The IFA assay was performed as described previously [31]. Briefly, acetone-fixed monolayers of Vero E6 cells infected with hantavirus were used as antigens. To detect the antibodies bound to antigen, FITC conjugated anti-rat, anti-human, and Protein A were used, as described previously [13]. These serum samples (1:100 dilution) showing characteristic fluorescence in infected Vero cells, but that were negative with uninfected Vero cells, were regarded as positive.

PCR, nucleotide sequencing, and phylogenetic analysis: Total RNA isolated from rat lung tissue was used to produce hantavirus-specific cDNA, as described previously [30]. Nucleotides (nt) 2000–2300 of the M genome segment were amplified using the primers SEOMF1936, (5'-gtggactcttct-tctcattatt-3') and SEOMR2353 (5'-tgggcaatctggggggttg-catg-3'). Similarly, nt 2000–3101 were amplified from the M genome segment using the primers SEOMF1936 and M12 (5'f-AACCACTATGGCCACCTTTC-3'). All products of the expected size were purified using a PCR purification kit (Qiagen) and sequenced using the original PCR primers. Sequencing was performed using a BigDye Terminator Cycle Sequencing Kit (ver. 3.1; Perkin Elmer) with a model 3100 DNA Sequencing System (Perkin Elmer) by Hokkaido System Science Co., Ltd. The sequences were aligned using CLUSTALW and Genetyx Mac (ver. 13.0.6) with default parameters (gap insert penalty, -12; gap extend penalty, -4). In subsequent phylogenetic analyses, neighbor-joining (NJ) phylogenetic trees and bootstrap analyses were calculated by Genetyx Mac (ver. 13.0.6) and/or Phylip (ver. 3.65).

Species specification by sequencing of mitochondrial cytochrome b: Total DNA, isolated from rat lung tissue using DNAZOL Reagent (Invitrogen), was subjected to PCR to amplify the entire coding region of cytochrome *b* (*cytb*; 1140 bp). The products were subsequently sequenced, as previously described [28].

RESULTS

Twelve of 837 human serum samples were positive by all three tests, ELISA, IFA, and Western blot, and were regarded as hantavirus-infected cases (Table 1). None of the samples were positive for PUUV or TPMV by ELISA (data not shown). Additionally, none of the samples contained IgM antibodies against HTNV (data not shown). The positive samples were collected in the provinces of Ha Nam, Thanh Hoa, and Hai Phong (Fig. 1). The positive rate among the febrile patients (2.3%) was roughly twice that among the healthy donors (1.1%).

ELISA was used to estimate the serotypes of the Vietnamese hantaviruses in the positive cases (Table 3). Two of the four positive samples from Thanh Hoa Province also showed a clear SEOV infection pattern. These results indicate the presence of an SEOV, whose rodent host belonged to the genus *Rattus* in the Hai Phong Port area and Thanh Hoa Province. However, the other two samples, 40B and

Table 3. Serotyping of antibody-positive human sera found in Vietnam by ELISA

Location	Serum ID	Status	ELISA OD				Determination
			rN	HTNV50	SEOV50	THAIV50	
Hai Phong	192	H	1.343	0.252	0.636	0.286	SEOV
Hai Phong	198	H	0.745	0.372	0.806	0.392	SEOV
Hai Phong	258	H	0.572	0.002	0.413	0.026	SEOV
Hai Phong	322	H	0.572	0.017	0.123	0.024	SEOV
Hai Phong	366	H	1.066	–0.002	0.413	0.026	SEOV
Ha Nam	75	P	0.370	–0.007	–0.017	–0.024	NI
Ha Nam	12	H	0.800	0.005	–0.028	–0.038	NI
Ha Nam	33	H	0.424	0.046	0.061	–0.041	NI
Thanh Hoa	63	P	0.343	0.035	0.240	0.032	SEOV
Thanh Hoa	38	P	0.317	0.106	0.188	0.044	SEOV
Thanh Hoa	40B	P	0.325	0.098	0.104	–0.002	NI
Thanh Hoa	149	P	0.298	0.089	0.113	–0.021	NI
HTNV antibody-positive			1.166	1.177	0.204	0.130	HTNV
SEOV antibody-positive			1.373	0.476	1.086	0.569	SEOV
THAIV antibody-positive			1.022	0.343	0.316	0.853	THAIV
Negative human sera-1			–0.008	–0.019	–0.013	–0.013	Negative
Negative human sera-3			–0.018	–0.019	–0.023	–0.009	Negative

Status H, healthy; status P, patient; NI, not identified.

149, showed virtually identical OD values, based on ELISA, when HTNV50, SEOV50, and THAIV50 were used as the serotyping antigens. Additionally, three of the positive samples from Ha Nam Province showed low OD values with three of the serotyping antigens, although they reacted strongly to full-length rN antigen from HTNV.

As shown in Table 2, 38 (8.6%) of the animal samples were antibody-positive in the three tests, ELISA, IFA, and Western blot. All but two of the positive samples were from *R. norvegicus*. The highest positive rate (33.9%) was observed among *R. norvegicus* captured at Hai Phong Port, in a warehouse. Two *R. rattus* captured in the warehouse were also antibody-positive. All of the sera were antibody-negative for PUUV, based on ELISA (data not shown). These results suggest that in Vietnam, hantavirus exists primarily in the brown rat (*R. norvegicus*).

The two antibody-positive *R. rattus* captured in the Hai Phong Port warehouse were classified as *R. rattus flavipectus*, based on morphological characteristics. To determine their classification genetically, the *cytb* gene from the mitochondrial DNA of the rats was sequenced and compared to representative sequences from other Rattus spp. [28]. As shown in Fig. 2A, the sequences obtained from three rats [all *R. rattus flavipectus*: #42 and #82 (antibody-positive) and #72 (antibody-negative)] were of the same lineage as those obtained from *R. tanezumi*, captured in Japan and China.

Among the seropositive brown rats (*R. norvegicus*) captured in Haibatrung and Hai Phong Port, 2 of 7 and 7 of 9 lung specimens, respectively, from the seropositive rats were positive for virus RNA, based on PCR. However, no part of the hantavirus genome was amplified from lung tissue collected from seropositive *R. rattus flavipectus* (*R. tanezumi*) captured at Hai Phong Port, although the two species of Rattus were found in the same warehouse as the positive *R. norvegicus*.

Among the PCR-positive specimens obtained from *R. norvegicus* captured in Hanoi Haibatrung District and the Hai Phong Port area, two and four specimens, respectively, were selected and the partial nucleotide sequences of their M genomic segments were compared. First, the Vietnamese hantaviruses were compared, based on short sequences from part of the Gc coding region, to various hantavirus strains. As shown in the phylogenetic tree drawn using 271nt of the M genomic segment (Fig. 2B), the Vietnamese hantaviruses were all found to belong to the SEOV clade. The Vietnamese viruses and Indonesian SEOV (Jakarta) [21] formed one group within the SEOV clade; thus, SEOV strains originating from Southeast Asia form a distinct lineage among SEOVs. To confirm the relationship among the Vietnamese and other SEOVs, a phylogenetic tree was drawn based on a longer sequence. As shown in Fig. 2C, both of the Vietnamese SEOVs obtained from Hai Phong and Hanoi Haibatroung District belong to the same SEOV cluster. In this tree, strain B1, which was isolated in Osaka, Japan, was located outside of the Vietnamese virus, alongside other Japanese strains obtained from Hokkaido in northern Japan, and it made a distinct cluster with the Korean SEOV. Another distinct cluster consisted of the Chinese SEOV.

DISCUSSION

Through serological examination of human sera for hantavirus infection, 12 antibody-positive sera were found from northern Vietnam. These results indicated that hantaviruses related to HTNV/SEOV are currently circulating in Vietnam. Roughly, a two-fold higher antibody positive rate was obtained among FUO patient sera compared with those of healthy donors. However, no statistically significant differ-

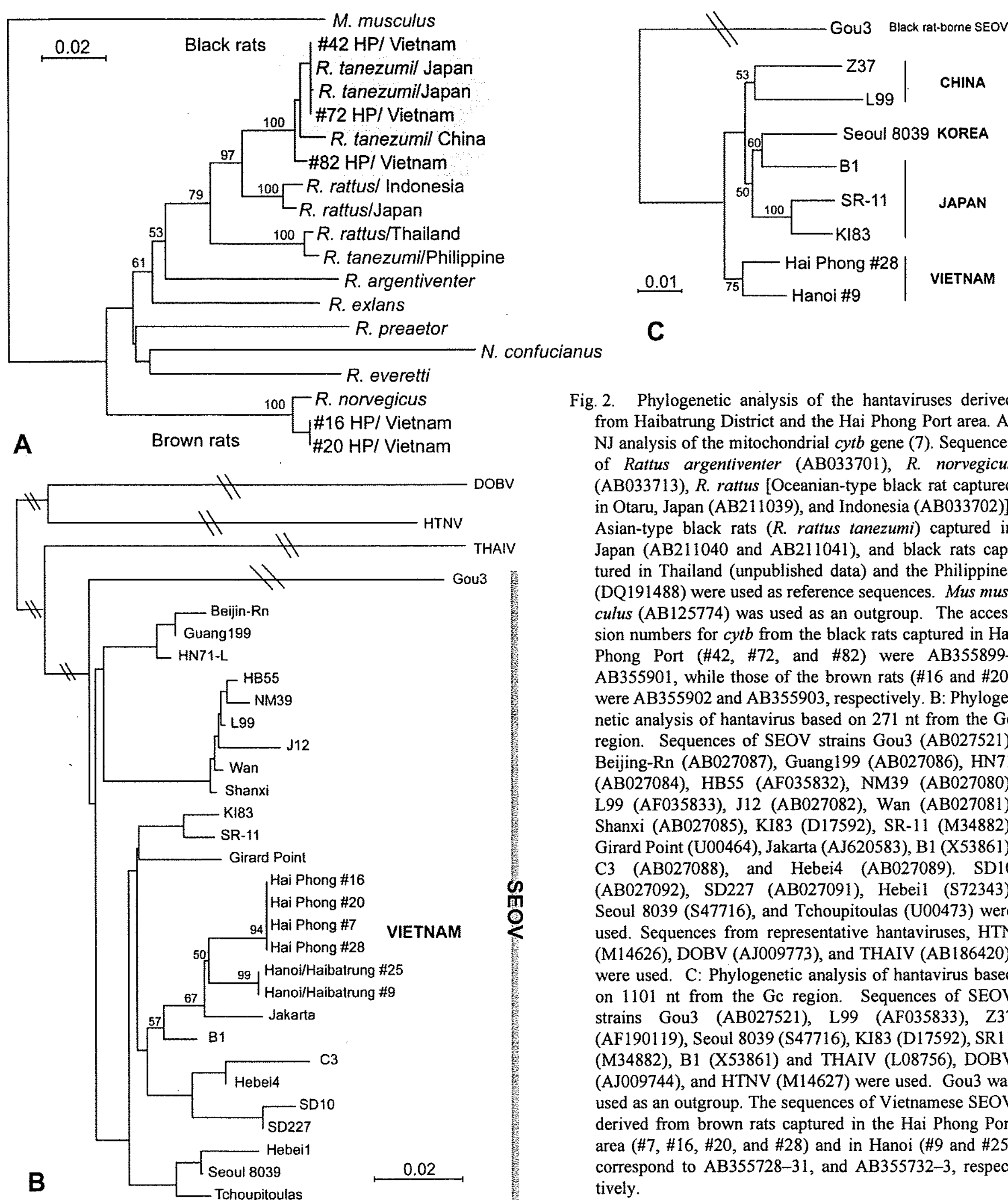

Fig. 2. Phylogenetic analysis of the hantaviruses derived from Haibatrung District and the Hai Phong Port area. A: NJ analysis of the mitochondrial *cytb* gene (7). Sequences of *Rattus argentiventer* (AB033701), *R. norvegicus* (AB033713), *R. rattus* [Oceanian-type black rat captured in Otaru, Japan (AB211039), and Indonesia (AB033702)], Asian-type black rats (*R. rattus tanezumi*) captured in Japan (AB211040 and AB211041), and black rats captured in Thailand (unpublished data) and the Philippines (DQ191488) were used as reference sequences. *Mus musculus* (AB125774) was used as an outgroup. The accession numbers for *cytb* from the black rats captured in Hai Phong Port (#42, #72, and #82) were AB355899–AB355901, while those of the brown rats (#16 and #20) were AB355902 and AB355903, respectively. B: Phylogenetic analysis of hantavirus based on 271 nt from the Gc region. Sequences of SEOV strains Gou3 (AB027521), Beijing-Rn (AB027087), Guangl99 (AB027086), HN71 (AB027084), HB55 (AF035832), NM39 (AB027080), L99 (AF035833), J12 (AB027082), Wan (AB027081), Shanxi (AB027085), KI83 (D17592), SR-11 (M34882), Girard Point (U00464), Jakarta (AJ620583), B1 (X53861), C3 (AB027088), and Hebei4 (AB027089). SD10 (AB027092), SD227 (AB027091), Hebei1 (S72343), Seoul 8039 (S47716), and Tchoupitoulas (U00473) were used. Sequences from representative hantaviruses, HTN (M14626), DOBV (AJ009773), and THAIV (AB186420), were used. C: Phylogenetic analysis of hantavirus based on 1101 nt from the Gc region. Sequences of SEOV strains Gou3 (AB027521), L99 (AF035833), Z37 (AF190119), Seoul 8039 (S47716), KI83 (D17592), SR11 (M34882), B1 (X53861) and THAIV (L08756), DOBV (AJ009744), and HTNV (M14627) were used. Gou3 was used as an outgroup. The sequences of Vietnamese SEOV derived from brown rats captured in the Hai Phong Port area (#7, #16, #20, and #28) and in Hanoi (#9 and #25) correspond to AB355728–31, and AB355732–3, respectively.

ence was shown between healthy and patient groups in a χ^2 test or Fisher test. Additionally, because no hantavirus-IgM antibody-positive serum was detected from IgG-positive sera, the relationship between FUO and hantavirus infection remains unclear. Positive sera from Ha Nam and two from Thanh Hoa could not be serotyped using the three serotyping antigens, HTNV50, SEOV50, and THAIV50. These positive human sera strongly reacted with rNP-HTNV, but were negative with PUUV (data not shown). Thus, these cases were considered to be infected with a novel hantavi-

rus, which might be related to Murinae-rodent associated hantaviruses, such as HTNV, SEOV, and THAIV. Previous studies have shown that hantaviruses belonging to three Murinae-associated species (HTNV, SEOV, THAIV) exist in East and Southeast Asia [10]; thus, serotyping antigens capable of distinguishing these three hantavirus species were used. All of the antibody-positive samples from the Hai Phong Port area showed higher OD values to the SEOV50 antigen than the other serotyping antigens (HTNV50 and THAIV50; Table 3). Thus, they were considered to be infected with SEOV. Generally in the port area, the brown rat (*R. norvegicus*) was found as the host rodent for SEOV. Also, in the Hai Phong Port area, antibody-positive brown rats and black rats were found (Table 2). To identify the host-virus relationship, the mitochondrial *cytb* gene and hantavirus genome from lung tissues of rats were examined. As shown in Table 2, the hantavirus genome was amplified from antibody-positive brown rats, but not from lung tissue of black rats. Further, the sequences were identified as brown rat-borne SEOV, different to the strain Gou3 sequence, which was a black rat-borne SEOV in China (30) (Fig. 2B, 2C). These results suggested that in the Hai Phong Port area, SEOV has spread from brown rats to black rats. These brown rats may also be the source of SEOV infection in human port workers in Hai Phong port. Also, the SEOVs circulating in Hai Phong port belonged to a distinct Vietnamese SEOV clade, with the SEOV derived from the Hanoi Haibatrung district.

Phylogenetic analysis of the *cytb* gene of rats also revealed at least three varieties among the black rats (*R. rattus*) in Asia: *R. tanezumi* (Oceanian black rat), *R. rattus* in Indonesia and Japan (European-type black rat), and *R. rattus* in the Philippines and Thailand. The black rats, classified to *R. rattus flavipectus* morphologically, that were captured in Hai Phong port, were classified as *R. tanezumi* by the *cytb* gene system. Novel hantaviruses were recently detected in black rats from China [30] and Cambodia [22]. Thus, to examine the relationship between hantaviruses and their reservoir rodents, rodent classification using the *cytb* gene system should be adopted.

None of the *R. rattus* (*tanezumi*) captured in Hai Phong Port warehouse were positive by PCR, although the *R. norvegicus* captured in the same warehouse were highly PCR-positive. This different PCR positivity between *R. norvegicus* and *R. rattus* (*R. tanezumi*) suggested that the principal host for Vietnamese hantavirus is *R. norvegicus*. *R. rattus* (*R. tanezumi*) was probably transiently infected, via a spillover event from *R. norvegicus*. Primers used in this study were also compatible with the *R. tanezumi*-borne virus, strain Gou3, from a previous report [30]. However, the possibility that this primer set was not able to amplify the *R. tanezumi*-borne virus gene found in Hai Phong port still remains.

As shown in Table 3, three of the positive samples from Ha Nam Province showed low OD values with the three serotyping antigens, although they reacted strongly to full-length rN antigen from HTNV. The low reactivity of the three Ha Nam cases, as well as the 2 unserotyped cases from Thanh Hoa, suggests the possibility of the existence of a novel species of hantavirus. As all of the positive sera were antibody-negative for PUUV, the novel species may be a Murinae-associated hantavirus.

Throughout the serological surveillance of small animals, numerous *S. murinus*, the natural host for TPMV [5], were captured. The presence of anti-TPMV antibodies was also examined by ELISA; however, all of the samples from the rats and *S. murinus* were negative. Recently, genetically distinct rodent-borne hantaviruses were detected in small mammals belonging to various species of Soricomorpha worldwide [1, 2, 12, 24–26]. Because these hantaviruses are phylogenetically distinct from TPMV, the antigenicity of TPMV may also differ from that in other soricomorph-borne hantaviruses. Thus, further serological surveillance for TPMV and other hantaviruses, including soricomorph-borne hantaviruses, is necessary.

In conclusion, we found that SEOV is circulating in northern Vietnam, in both humans and rodents; however, the consequence of SEOV infection as a cause of HFRS remains unclear. The Vietnamese SEOV is phylogenetically distinct from SEOVs originating in other regions, suggesting that Southeast Asian SEOVs form a separate cluster. As the existence of novel hantaviruses was also suggested, additional epidemiological and epizootiological studies are required to clarify the variation in, and distribution of, hantaviruses in East and Southeast Asia.

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REFERENCES

1. Arai, S., Bennett, S. N., Sumibcay, L., Cook, J. A., Song, J. W., Hope, A., Parmenter, C., Nerurkar, V. R., Yates, T. L. and Yanagihara, R. 2008. Phylogenetically distinct hantaviruses in the masked shrew (*Sorex cinereus*) and dusky shrew (Sorex monticolus) in the United States. *Am. J. Trop. Med. Hyg.* **78**: 348–351.
2. Arai, S., Song, J. W., Sumibcay, L., Bennett, S. N., Nerurkar, V. R., Parmenter, C., Cook, J. A., Yates, T. L. and Yanagihara, R. 2007. Hantavirus in northern short-tailed shrew, United States. *Emerg. Infect. Dis.* **13**: 1420–1423.
3. Araki, K., Yoshimatsu, K., Ogino, M., Ebihara, H., Lundkvist, A., Kariwa, H., Takashima, I. and Arikawa, J. 2001. Truncated hantavirus nucleocapsid proteins for serotyping Hantaan, Seoul, and Dobrava hantavirus infections. *J. Clin. Microbiol.* **39**: 2397–2404.
4. Brummer-Korvenkontio, M., Vaheri, A., Hovi, T., von Bons-

dorff, C. H., Vuorimies, J., Manni, T., Penttinen, K., Oker-Blom, N. and Lahdevirta, J. 1980. Nephropathia epidemica: detection of antigen in bank voles and serologic diagnosis of human infection. *J. Infect. Dis.* **141**: 131–134.

5. Carey, D. E., REuben, R., Panicker, K. N., Shope, R. E. and Myers, R. M. 1971. Thottapalayam virus: A presumptive arbovirus isolated from a shrew in India. *Indian J. Med. Res.* **59**: 1758–1760.
6. Chandy, S., Yoshimatsu, K., Boorugu, H. K., Chrispal, A., Thomas, K., Peedicyil, A., Abraham, P., Arikawa, J. and Sridharan, G. 2009. Acute febrile illness caused by hantavirus: serological and molecular evidence from India. *Trans. R. Soc. Trop. Med. Hyg.* **103**: 407–412.
7. Chinen, A. A., Suzuki, H., Aplin, K. P., Tsuchiya, K. and Suzuki, S. 2005. Preliminary genetic characterization of two lineages of black rats (*Rattus rattus sensu lato*) in Japan, with evidence for introgression at several localities. *Genes Genet. Syst.* **80**: 367–375.
8. Elliott, R. M. 1996. The Bunyaviridae. Plemun Press, New York.
9. Elwell, M. R., Ward, G. S., Tingpalapong, M. and LeDuc, J. W. 1985. Serologic evidence of Hantaan-like virus in rodents and man in Thailand. *Southeast Asian J. Trop. Med. Public Health* **16**: 349–354.
10. Kariwa, H., Yoshimatsu, K. and Arikawa, J. 2007. Hantavirus infection in East Asia. *Comp. Immunol. Microbiol. Infect. Dis.* **30**: 341–356.
11. Kitamura, T., Morita, C., Komatsu, T., Sugiyama, K., Arikawa, J., Shiga, S., Takeda, H., Akao, Y., Imaizumi, K., Oya, A., Hashimoto, N. and Urasawa, S. 1983. Isolation of virus causing hemorrhagic fever with renal syndrome (HFRS) through a cell culture system. *Jap. J. Med. Sci. Biol.* **36**: 17–25.
12. Klempa, B., Fichet-Calvet, E., Lecompte, E., Auste, B., Aniskin, V., Meisel, H., Barriere, P., Koivogui, L., ter Meulen, J. and Kruger, D. H. 2007. Novel hantavirus sequences in Shrew, Guinea. *Emerg. Infect. Dis.* **13**: 520–522.
13. Lee, B. H., Yoshimatsu, K., Araki, K., Ogino, M., Okumura, M., Tsuchiya, K., Kariwa, H. and Arikawa, J. 2003. Detection of antibody for the serodiagnosis of hantavirus infection in different rodent species. *Arch. Virol.* **148**: 1885–1897.
14. Lee, H. W. 1999. Epidemiology and Epizootiology, pp. 40–48. *In*: Manual of Hemorrhagic Fever with Renal Syndrome and Hantavirus Pulmonary Syndrome (Lee, H. W., Calisher, C. and Schmaljohn, C. S. eds.). WHO Collaborating Center for Virus Reference and Research, Seoul.
15. Lee, H. W., Lee, P. W. and Johnson, K. M. 1978. Isolation of the etiologic agent of Korean hemorrhagic fever. *J. Infect. Dis.* **137**: 298–308.
16. Miyamoto, H., Kariwa, H., Araki, K., Lokugamage, K., Hayasaka, D., Cui, B. Z., Lokugamage, N., Ivanov, L. I., Mizutani, T., Iwasa, M. A., Yoshimatsu, K., Arikawa, J. and Takashima, I. 2003. Serological analysis of hemorrhagic fever with renal syndrome (HFRS) patients in Far Eastern Russia and identification of the causative hantavirus genotype. *Arch. Virol.* **148**: 1543–1556.
17. Nakamura, I., Yoshimatsu, K., Lee, B.-H., Okumura, M., Taruishi, M., Araki, K., Kariwa, H., Takashima, I. and Arikawa, J. 2008. Development of a serotyping ELISA system for Thailand virus infection. *Arch. Virol.* **153**: 1537–1542.
18. Okumura, M., Yoshimatsu, K., Kumperasart , S., Nakamura, I., Ogino, M., Taruishi, M., Sungdee, A., Pattamadilok, S., Ibrahim, I. N., Erlina, S., Agui, T., Yanagihara, R. and Arikawa, J. 2007. Development of serological assays for Thottapalayam virus, an insectivore-borne Hantavirus. *Clin. Vaccine Immunol.* **14**: 173–181.
19. Pattamadilok, S., Lee, B. H., Kumperasart, S., Yoshimatsu, K., Okumura, M., Nakamura, I., Araki, K., Khoprasert, Y., Dangsupa, P., Panlar, P., Jandrig, B., Kruger, D. H., Klempa, B., Jakel, T., Schmidt, J., Ulrich, R., Kariwa, H. and Arikawa, J. 2006. Geographical distribution of hantaviruses in Thailand and potential human health significance of Thailand virus. *Am. J. Trop. Med. Hyg.* **75**: 994–1002.
20. Plyusnin, A., Vapalahti, O. and Lundkvist, A. 1996. Hantaviruses: genome structure, expression and evolution. *J. Gen. Virol.* **77**: 2677–2687.
21. Plyusnina, A., Ibrahim, I. N., Winoto, I., Porter, K. R., Gotama, I. B., Lundkvist, A., Vaheri, A. and Plyusnin, A. 2004. Identification of Seoul hantavirus in Rattus norvegicus in Indonesia. *Scand. J. Infect. Dis.* **36**: 356–359.
22. Reynes, J. M., Soares, J. L., Hue, T., Bouloy, M., Sun, S., Kruy, S. L., Flye Sainte Marie, F. and Zeller, H. 2003. Evidence of the presence of Seoul virus in Cambodia. *Microbes Infect.* **5**: 769–773.
23. Schmaljohn, C. and Hjelle, B. 1997. Hantaviruses – a Global Disease Problem. *Emerg. Infect. Dis.* **3**: 95–104.
24. Song, J. W., Baek, L. J., Schmaljohn, C. S. and Yanagihara, R. 2007. Thottapalayam virus, a prototype shrewborne hantavirus. *Emerg. Infect. Dis.* **13**: 980–985.
25. Song, J. W., Gu, S. H., Bennett, S. N., Arai, S., Puorger, M., Hilbe, M. and Yanagihara, R. 2007. Seewis virus, a genetically distinct hantavirus in the Eurasian common shrew (*Sorex araneus*). *Virol. J.* **4**: 114.
26. Song, J. W., Kang, H. J., Song, K. J., Truong, T. T., Bennett, S. N., Arai, S., Truong, N. U. and Yanagihara, R. 2007. Newfound hantavirus in Chinese mole shrew, Vietnam. *Emerg. Infect. Dis.* **13**: 1784–1787.
27. Suputthamongkol, Y., Nitatpattana, N., Chayakulkeeree, M., Palabodeewat, S., Yoksan, S. and Gonzalez, J. P. 2005. Hantavirus infection in Thailand: First clinical case report. *Southeast Asian J. Trop. Med. Public Health* **36**: 217–220.
28. Suzuki, H., Tsuchiya, K. and Takezaki, N. 2000. A molecular phylogenetic framework for the Ryukyu endemic rodents *Tokudaia isimensis* and *Diplothrix legata*. *Mol. Phylogenet. Evol.* **15**: 15–24.
29. Takakura, A., Goto, K., Itoh, T., Yoshimatsu, K., Takashima, I. and Arikawa, J. 2003. Establishment of an Enzyme-linked immunosorbent assay for detection of hantavirus antibody of rats using a recombinant of nucleocapsid protein expressed in Escherichia coli. *Exp. Anim.* **52**: 25–30.
30. Wang, H., Yoshimatsu, K., Ebihara, H., Ogino, M., Araki, K., Kariwa, H., Wang, Z., Luo, Z., Li, D., Hang, C. and Arikawa, J. 2000. Genetic diversity of hantaviruses isolated in china and characterization of novel hantaviruses isolated from *Niviventer confucianus* and *Rattus rattus*. *Virology* **278**: 332–345.
31. Yoshimatsu, K., Arikawa, J. and Kariwa, H. 1993. Application of a recombinant baculovirus expressing hantavirus nucleocapsid protein as a diagnostic antigen in IFA test: cross reactivities among 3 serotypes of hantavirus which causes hemorrhagic fever with renal syndrome (HFRS). *J. Vet. Med. Sci.* **55**: 1047–1050.
32. Yoshimatsu, K., Arikawa, J., Yoshida, R., Li, H., Yoo, Y. C., Kariwa, H., Hashimoto, N., Kakinuma, M. and Azuma, I. 1995. Production and application of recombinant hantavirus nucleocapsid protein expressed in silkworm larvae as a diagnostic antigen for laboratory rat. *Lab. Anim. Sci.* **45**: 647–651.

FULL PAPER *Public Health*

Epidemiological Study of Hantavirus Infection in the Samara Region of European Russia

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ABSTRACT. European Russia is a highly endemic area of hemorrhagic fever with renal syndrome (HFRS), a rodent-borne zoonotic disease, caused by hantaviruses. In total, 145 small mammals of four species (*Myodes glareolus, Apodemus flavicollis, A. agrarius*, and *A. uralensis*) were trapped in the Samara region of European Russia in August 2005 and examined for the presence of hantavirus (HV). Anti-HV antibodies were found in six of 68 (8.8%) *M. glareolus* and in one of 19 (5.3%) *A. flavicollis* by indirect immunofluorescent antibody assay (IFA). The Puumala virus (PUUV), which is one of the hantavirus species, was detected in the lungs of seven *M. glareolus* by RT-PCR. The virus S-segment was extremely similar (96.2% to 99.3%) to the sequence found in a fatal case of HFRS in the Samara region. Phylogenetic analyses of S and M segments showed that the Samara PUUVs form a cluster within the Russian Volga lineage and apparently differ from other European PUUVs. Anti-PUUV antibodies were found in blood sera from seven HFRS patients and from one undiagnosed patient from the Samara region, using IFA and an enzyme-linked immunosorbent assay (ELISA). These data suggest that the bank vole *M. glareolus* is a primary natural reservoir and vector for PUUV, which is the main causative agent of HFRS in humans in the Samara region.

KEY WORDS: epidemiology, hantavirus, hemorrhagic fever with renal syndrome, Puumala virus, rodents.

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Hantaviruses belong to the genus *Hantavirus*, within the family *Bunyaviridae*, and cause two forms of human illness. These zoonoses include hemorrhagic fever with renal syndrome (HFRS), which occurs in Asia and Europe and is caused by the Hantaan virus (HTNV), Seoul virus (SEOV), Puumala virus (PUUV), Dobrava-Belgrade virus (DOBV), and Amur virus (AMRV). The second form of illness is hantavirus (cardio) pulmonary syndrome (HPS), which occurs in the Americas and is caused by the Sin Nombre virus, Andes virus, and other hantaviruses [13, 28, 33]. Hantaviruses are transmitted by aerosolized excreta from their natural hosts, mainly rodents of the family *Muridae*. Particular hantavirus species are usually harbored by a single rodent species, or by a few closely related rodent species, which indicates co-evolution and co-speciation of hantaviruses with their hosts. The virus genome contains three segments of negative-stranded RNA: the large (L) segment encodes a viral RNA-dependent RNA polymerase, the medium (M) segment encodes a glycoprotein precursor, and the small (S) segment encodes a nucleocapsid protein (NP) [34].

In Russia, five viruses cause HFRS in humans: PUUV and DOBV cause HFRS in European Russia [41], and HTNV, SEOV, and AMRV cause HFRS in Far Eastern Russia [16, 19, 38, 46]. Sporadic HFRS cases, caused by PUUV and DOBV, were recently detected in the western Siberian regions of Russia [45]. The principal hosts for HTNV, AMRV, SEOV, and PUUV are *Apodemus agrarius, A. peninsulae, Rattus norvegicus*, and *Myodes glareolus*, respectively. In Europe, detailed phylogenetic analyses show that the DOBV strains from *A. flavicollis* form a separate evolutionary lineage (DOBV-Af) while strains from *A. agrarius* show higher levels of diversity. Strains from central Europe and central European Russia form the DOBV-Aa lineage, which is distinct from the Saaremaa strains from northeastern Europe [9, 10]. In southern Russia (Sochi district), a new DOBV variant (DOBV-Ap) found in *A. ponticus*, a novel hantavirus natural host, was identified as the causative agent of HFRS [11, 42]. In European Russia and Europe, although the DOBV strains from different *Apodemus* hosts share high amino acid sequence similarity, they can be distinguished in phylogenetic analyses as distinct lineages and seem to possess different virulence in humans as well as in an animal model [12]. The novel DOBV-Ap lineage associated with *A. ponticus*, emerging in an area south of European Russia, confirms the reputation of DOBV as being the most virulent of the European hantaviruses [11]. Rather unusually for hantaviruses, DOBV has already been found in three different Apodemus species. In addition to DOBV,

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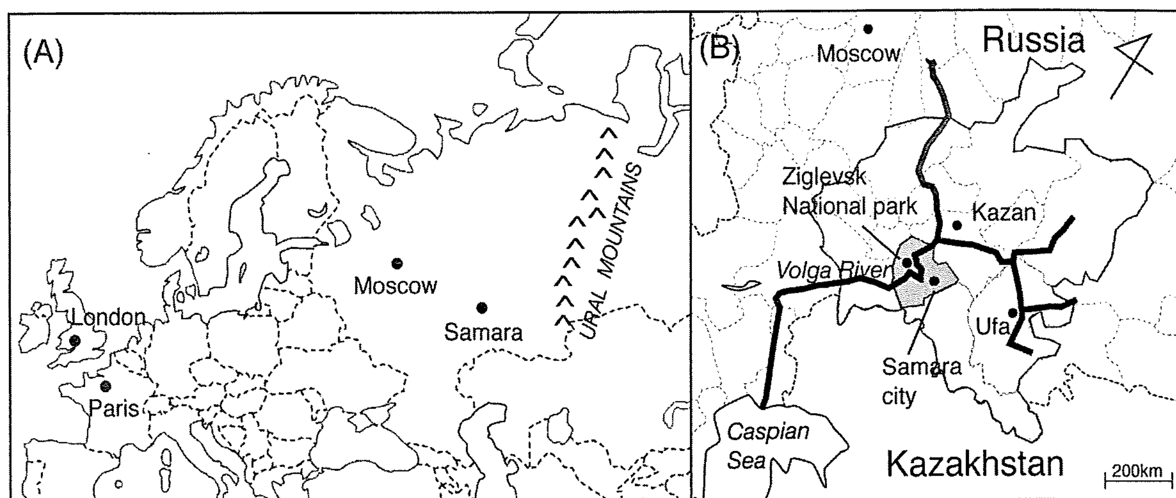

Fig. 1. Map of the survey site. (A) European Russia and European countries. (B) Samara region in the Volga River area; Samara region.

other hantaviruses are also harbored by more than one host species, for example, the Tula virus, which is carried by *Microtus arvalis, M. rossiaemeridionalis*, and *M. agrestis* [29, 35, 36] and SEOV, carried by *Rattus rattus* and *R. norvegicus* [14].

In Russia, HFRS has the highest incidence and morbidity among zoonotic virus infections in humans. The disease has been recognized in Russia since 1930 and has been included in the official reporting system of the Russian Ministry of Public Health since 1978. Of HFRS cases, 97% have been registered in European Russia, while only 3% have been registered in Asian (Far Eastern) Russia [40]. The results of the comparative analyses of clinical data from more than 5,000 HFRS cases from seven different endemic foci, using a common scheme of criteria of severity, indicated that three hantaviruses in Far Eastern Russia (HTNV, SEOV, AMRV) and two hantaviruses in European Russia (PUUV and DOBV) can produce three clinical forms of the disease: mild, moderate, and severe [5, 15]. The rates of severe HFRS caused by HTNV and AMRV in the Far Eastern regions and the DOBV-Ap lineage in southern Russia are significantly higher than the rate of severe HFRS caused by the PUUV, SEOV, and DOB-Aa lineages (Central European Russia). Since HFRS in European Russia is caused by several hantaviruses and the severity varies a great deal, it is inappropriate to use the term "nephropathia epidemica" for the disease. The term HFRS was recommended by the World Health Organization (WHO) Working Group in 1983 to unify the different names of similar clinical diseases in Russia, Europe and Asia [44].

There are approximately 6000 to 8000 clinical cases of HFRS every year, scattered throughout European Russia, that are caused primarily by PUUV. However, the morbidity rates in different administrative regions vary considerably. The highest incidence of HFRS occurs annually in the Ural Mountains and Volga River areas, where there are nine administrative regions with high HFRS morbidity in European Russia [40]. One of the regions in this territory is the Samara region, where we carried out an epizootiological and epidemiological survey in 2005. According to previous studies, most of the HFRS cases in this region seemed to be caused by PUUV infection. However, the characteristics of PUUVs in the region are not well documented and antibodies to HTNV were also detected in healthy blood donors in the region [1]. Therefore, the goals of this study were: (1) to investigate the epidemiological situationy of hantavirus infection both in rodents and humans in the Samara region; (2) to obtain genetic information for hantaviruses, and (3) to identify the epidemiological significance of rodent hosts and hantaviruses with respect to the morbidity of HFRS.

MATERIALS AND METHODS

Rodent survey and human sera: For this study we conducted epizootiological survey targeting rodents. Animals were captured using snap traps that were located in forests near the Zigulevsk National Park, west of the Volga River, and in suburbs of the city of Samara in August 2005 (Fig. 1). Rodent blood samples were collected using filter paper. After complete drying, the filter paper was immersed in 10× the volume of phosphate-buffered saline (PBS) at 4°C overnight. The eluted blood sample was heat-inactivated at 56°C for 30 min and centrifuged at 300 × g for 5 min. Thereafter, the supernatant was used as 1:10 diluted serum. The lungs, hearts, livers, kidneys, and spleens were collected from the captured rodents and stored at –80°C until use. Human blood sera were obtained from clinically diagnosed HFRS patients and patients with high fever of unknown etiologies. Blood sera were stored at –80°C until use.

Indirect immunofluorescent antibody assay (IFA): Anti-hantavirus antibodies in rodents and patients were detected

Table 1. Primers used for RT-PCR and sequencing of S and M genome segments of hantaviruses

Virus	Gene	Primer name	Primer sequence	Position
Puumala	S	sotkamo62Fw	tccaagaggatataacccgccat	62–84
		sotS172Fw	ctgcaagccaggcaacaaacagtgtcagca	172–201
		sotS593Fw	ctcagtccaccatgaag	593–609
		ufa97S850Fw	aagccagaagttaaacct	850–867
		samS1146Fw	atatttgcgccggacacaatc	1146–1166
		samS1286Fw	gtcactcatcgatcagaagg	1286–1305
		samS1552Fw	cagggaattactaatgacag	1552–1571
		sotS537Rv	ctcaaatgatgtgtcatcc	537–519
		sotS894Rv	gtctgccacatgatttttgtcaagcacatc	894–865
		samS1037Rv	aaytcagccatcccagcaac	1037–1018
		samS1500Rv	gataataataattgtcaaacccg	1500–1478
		PUU1801SRv	atcagcatgttgaggtagta	1801–1782
	M	Ufa97M1789F	tctcaatccatctgaggcaacaac	1789–1812
		sam94M2122f	gctatacatataggagacag	2122–2141
		sam94M2546F	actacttcagtcaaagtgtg	2546–2565
		sam94M2956F	gatttaagtgaaacaccatgcca	2956–2978
		sam94M3303F	tgatgatggtgcaccagag	3303–3321
		sam94M2019R	gtctcagcactagcagcccatac	2019–1997
		sam94M2317R	gcagtctgccaaggataagc	2317–2298
		sam94M2478R	ctggtataccttaaggacac	2478–2459
		sam94M2885R	ctaatgcacttgcagatatg	2885–2866
		sam94M3315R	tggtgcaccatcatcaaag	3315–3297
		Ufa97M3654R	ccaggcataatcggtatggggtaa	3654–3631
Dobrava	S	DOBS84Fw	caattggtgatagccaggcagaagg	84–108
		DOBS1012Rv	gccatgcctgcaatgaacaggcagg	1012–988

by IFA. Antigen slides were prepared using Vero E6 cells (ATCC No. CRL-1586) separately infected with the PUUV strain Sotkamo, the HTNV strain 76–118, and the SEOV strain SR-11. Serially diluted rodent blood sera (from 1:16 to 1:2048) were applied to the antigen slides which were then incubated at 37°C for 1 hr. After three washes with PBS, Protein G Alexa Fluor® 488 conjugate (Invitrogen, Carlsbad, CA, U.S.A.) or Alexa Fluor® 488 Goat Anti-Mouse IgG (Invitrogen) was spotted on to the slides. After incubation at 37°C for 1 hr, the slides were washed and observed by fluorescence microscopy. Scattered granular fluorescence in the cytoplasm of Vero E6 cells was considered as a positive reaction.

Enzyme-linked immunosorbent assay (ELISA): Anti-hantavirus IgG in patients was detected by ELISA [2, 20, 27]. Briefly, 96-well plates were coated with the monoclonal antibody (MAb) E5/G6 [26, 47] as a capture antibody and were treated with Block Ace (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) at 37°C for 1 hr for blocking. The Baculovirus-expressed recombinant hantavirus NP of PUUV, DOBV, or HTNV [2, 20] was then added to the plates and incubated at room temperature for 1 hr. Sera from clinically diagnosed HFRS patients were diluted to 1:200, added to the plates, and incubated at room temperature for 1 hr. Alkaline phosphatase-conjugated anti-human IgG (Sigma-Aldrich Cooperation, St. Louis, MO) was diluted to 0.5 μg/m*l*, added to the plates, and incubated at room temperature for 1 hr. Finally, p-nitrophenyl phosphate solution (Sigma-Aldrich) was added to the plates, which were incubated at room temperature for 1 hr, and absorbance values at 405 nm (A405) and 650 nm (A650) were measured using a spectrophotometer. The IgG-ELISA value was expressed as the value of A405 minus A650. Between the steps of the ELISA assay, the plates were washed three times with PBS containing 0.05% Tween 20 (PBST).

Anti-hantavirus IgM in patient sera was detected by μ-capture ELISA. Briefly, 96-well plates were coated with goat-anti human IgM (MP Biomedicals United States, Solon, OH) as the capture antibody. After blocking, 1:200 diluted patient sera were added to the plates and incubated at room temperature for 1 hr. Recombinant NP proteins of PUUV, DOBV, and HTNV were then added to the plates and incubated at room temperature for 1 hr. Biotinylated E5/G6 mAb and peroxidase-conjugated streptavidin (Sigma-Aldrich) were applied consecutively and incubated at room temperature for 1 hr. Thereafter, 3,3',5,5'-tetramethylbenzidine solution (Sigma-Aldrich) was added to the plates as a substrate. The colorimetric reaction was stopped by adding 50 μl of 0.5 M H_2SO_4. The IgM-ELISA value was calculated as described for the IgG-ELISA.

Reverse transcription-polymerase chain reaction (RT-PCR): Total RNA from the lung tissue of *M. glareolus* and *A. flavicollis* was extracted using ISOGEN (Nippon Gene Co., Ltd., Osaka, Japan). The RNA (5 μg) was reverse-transcribed using 200 units of Superscript II RNase H-reverse transcriptase (Invitrogen Corporation) and 500 *ng* random primers (Invitrogen) according to the manufacturer's

instructions. The S and M hantavirus segments were amplified by PCR using Platinum® Taq DNA polymerase High Fidelity (Invitrogen). The thermal conditions for PCR were 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 50°C to 60°C (depending on the primers used) for 30 sec, and 68°C for 2 min. The primers used for PCR are listed in Table 1. Primers SotS172Fw and SotS894Rv were used to detect PUUV, and DOBS84Fw and DOBS1012Rv were used to detect DOBV (Table 1).

Sequencing of hantavirus genome segments: The amplified DNA fragments were separated using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI). The purified DNA fragments were sequenced using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) according to the manufacturer's instructions. Primers used for amplification and sequencing are listed in Table 1.

Phylogenic analysis: The ClustalX version 1.83 (available from the URL: ftp://ftp.ebi.ac.uk/pub/software/clustalw2) was used to generate the alignments, and phylogenetic trees were formed by the neighbor-joining method [39]. The reliability of the dendrogram was evaluated by 1,000 bootstrap replicates.

RESULTS

Rodent survey: We carried out an epizootiological survey and captured 145 rodents in the Samara region. The rodent species included 68 *M. glareolus*, 19 *A. flavicollis*, 21 *A. agrarius*, and 37 *A. uralensis*. Six *M. glareolus* (8.8%) and one *A. flavicollis* (5.3%) harbored anti-hantavirus antibodies (Table 2). No antibodies were detected in *A. agrarius* or *A. uralensis*. Antibody titers to PUUV, measured by IFA, in *M. glareolus* ranged from 1:256 to 1:1024, and showed titers more than two times higher than antibody titers to HTNV or SEOV. One *A. flavicollis* had antibodies to both HTNV and SEOV at 1:16 (Table 3) but was negative for PUUV. Total RNA samples extracted from the lungs of 68 *M. glareolus* and 19 *A. flavicollis* were subjected to RT-PCR. Hantavirus genes were detected in four seropositive, and three seronegative *M. glareolus* (Tables 2 and 3), whereas no hantavirus genes were detected in *A. flavicollis*.

Sequencing of hantavirus genes: Partial S- and M-segments were sequenced from RT-PCR-positive *M. glareolus*. The viruses carried by *M. glareolus* (animal numbers: 6, 49, 68, 94, 112, 128 and 147) were named Samara_6/CG/2005 (Sam6); Samara_49/CG/2005 (Sam49); Samara_68/CG/2005 (Sam68); Samara_94/CG/2005 (Sam94); Samara_112/CG/2005 (Sam112); Samara_128/CG/2005 (Sam128) and Samara_147/CG/2005 (Sam147), respectively. The sequences determined and used in this study are listed in Table 4.

The S-segment sequences of viruses in the Samara region, which were similar to those of the PUUV strains Kazan, CG1820, and DTK/Ufa-97, identified near the Samara region within the Volga river area, demonstrated

Table 2. Detection of anti-hantavirus antibodies and virus RNA among captured rodents in the Samara region

Rodent species	Number of rodents	Seroprevalence (%)	Detection rate of Virus RNA (%)[a)]
M. glareolus	68	6/68 (8.8)	7/68 (10.1)
A. flavicollis	19	1/19 (5.3)	0/19 (0.0)
A. agrarius	21	0/21 (0)	ND[b)]
A. uralensis	37	0/37 (0)	ND
Total	145	7/145 (4.8)	

a) Virus RNA was detected from rodent lungs by RT-PCR.
b) ND: not done.

Table 3. Rodents with anti-hantavirus antibodies and virus RNA

Rodent species	Point of capture	Animal No.	IFA titer			Virus RNA[a)]
			PUUV	HTNV	SEOV	RT-PCR
M. glareolus	Suburb of Samara	66	256	<16	<16	–[b)]
		137	512	256	128	–
		68	1024	<16	<16	+[c)]
		147	1024	64	64	+
		94	<16	<16	<16	+
	Zigulevsk national park	6	<16	<16	<16	+
		49	<16	<16	<16	+
		112	512	64	64	+
		128	256	<16	<16	+
A. flavicollis	Suburb of Samara	59	<16	16	16	–

a) Virus RNA was detected from rodent lungs by RT-PCR. b) negative. c) positive.

Table 4. Hantavirus sequences used in this study

Virus name	Strain	Source	Country/ Region	Accession No.	
				S segment	M segment
Hantaan	76–118	*Apodemus*	Korea	M14626	M14627
Tula	Moravia/ 5302v/95	*Microtus arvalis*	Czech Republic	Z69991	Z69993
Khabarovsk	Ls136V	*Microtus fortis*	Russia	AJ011646	
Hokkaido	Kamiiso	*Myodes rufocanus*	Japan	AB010730	
	Tobetsu	*M.rufocanus*	Japan	AB010731	
Puumala	Balkan-1	*Myodes glareolus*	Balkan	AJ314600	
	Balkan-2	*M. glareolus*	Balkan	AJ314601	
	Klippitztoerl	*M. glareolus*	Austria	AJ888751	
	Ernstbrunn	*M. glareolus*	Austria	AJ888752	
	Opina	*M. glareolus*	Slovakia	AF294652	
	Mignovillard	*M. glareolus*	France	AM695638	
	CG13891	*M. glareolus*	Belgium	U22423	U22418
	Cg-Erft	*M. glareolus*	Belgium	AJ238779	AJ238778
	Thuin	*M. glareolus*	Belgium	AJ277030	
	Couvin	*M. glareolus*	Belgium	AJ277034	AJ277040
	Eidsvoll	*M. glareolus*	Norway	AJ223368	
		M. glareolus	Norway	AJ223369	
	Solleftea	*M. glareolus*	Norway	AJ223376	
		M. glareolus	Norway	AJ223377	
	Fin	*M. glareolus*	Denmark	AJ238791	
		M. glareolus	Denmark	AJ278092	
		M. glareolus	Denmark	AJ278093	
	Umea/hu	HFRS patient	Sweden	AY526219	AY526218
	Vindeln	*M. glareolus*	Sweden	Z48586	Z49214
	Vranica	*M. glareolus*	Sweden	U14137	U14136
	Virrat	*M. glareolus*	Finland	Z69985	
	Evo	*M. glareolus*	Finland	Z30702	
		M. glareolus	Finland	Z30703	
	Pallasjarvi	*M. glareolus*	Finland	AJ314597	
	Kolodozero	*M. glareolus*	Finland	AJ238789	
	Karhumaki	*M. glareolus*	Finland	AJ238788	
	Gomselga	*M. glareolus*	Finland	AJ238790	
	Sotkamo	*M. glareolus*	Finland	X61035	X61034
	1324Cg/79	*M. glareolus*	Finland	Z46942	

94% to 95% nucleotide similarity identity and 98% similarity identity in the deduced amino acid sequences (Table 5). The homology identities of the nucleotide and amino acid sequences between the viruses in the Samara and Scandinavian PUUVs (strains Sotkamo and Umea/Hu) were 85% and 96%, respectively.

In addition, comparisons of S-segments between sequences identified from the Zigulevsk National Park (Sam6, Sam49, Sam112, and Sam128) and F-s808, which was detected from a fatal case of HFRS in the Samara region, showed 99.3% and 100.0% homology at the nucleotide and amino acid levels, respectively (Table 5).

Phylogenetic analysis: Phylogenetic analyses of hantavirus S- and M-segments were performed (Fig. 2). The result clearly indicated that the hantaviruses detected in *M. glareolus* belong to the Volga River area lineage of PUUV. This lineage consists of viruses originating from the Samara region (Sam6, Sam49, Sam68, Sam94, Sam112, Sam128, Sam147, F-s808); Tatarstan (Kazan); Bashkortostan (CG17/Bashkiria-2001, CG1820, K27, DTK/Ufa-97); Saratov (P360) and Udmurt (Udmurtia/894Cg/91, Udmurtia/444Cg/88). They occupied one cluster and were distant from the viruses derived from other Russian regions (such as Omsk) and European countries (Fig. 2). The virus clusters were formed according to the geographical origin of the viruses.

Serological analysis of patient sera: To examine the causative agents of HFRS, serological analyses were performed on 12 blood sera from seven patients from the Samara region who were clinically diagnosed with HFRS, as well as on nine sera from six patients with high fevers of unknown etiologies. Reactivities of sera to PUUV, SEOV, and HTNV were analyzed by IFA (Table 6). Sera from six HFRS patients (Patients 1, 2, 3, 4, 6, and 7) and from one of the unknown fever patients (Patient 11) contained anti-hantavirus antibodies. In all positive sera, IFA titers to PUUV were at least 16 times higher than titers to SEOV and HTNV. In patient numbers 1, 2 and 3, titers of convalescent sera to PUUV were higher than those of acute phase sera

Table 4. continued. Hantavirus sequences used in this study

Virus name	Strain	Source	Country/ Oblast/Region	Accession No. S segment	Accession No. M segment
Puumala	CG144	*M. glareolus*	Russia/ Omsk	AF367064	
	CG215	*M. glareolus*	Russia/ Omsk	AF367066	
	CG222	*M. glareolus*	Russia/ Omsk	AF360067	
	Baltic	*M. glareolus*	Baltic	AJ314598	
		M. glareolus	Baltic	AJ314599	
	CG17	*M. glareolus*	Russia/ Bashkortostan	AF442613	AF442614
	CG1820	*M. glareolus*	Russia/ Bashkortostan	M32750	M29979
	K27		Russia/ Bashkortostan	L08804	L08754
	P360		Russia/ Bashkortostan	L11347	L08755
	DTK/Ufa-97	HFRS patient	Russia/ Bashkortostan	AB297665	AB297666
	Udmurtia	*M. glareolus*	Russia/Udmurt	Z21497	
		M. glareolus	Russia/Udmurt	Z30706	
	Kazan	*M. glareolus*	Russia/ Tatarstan	Z84204	Z84205
	Samara_6/ CG/2005	*M. glareolus*	Russia/Samara Zigulevsk	AB433842	AB433849
	Samara_49/ CG/2005	*M. glareolus*	Russia/Samara Zigulevsk	AB433843	AB433850
	Samara_112/CG/2005	*M. glareolus*	Russia/Samara Zigulevsk	AB433846	AB433853
	Samara_128/CG/2005	*M. glareolus*	Russia/Samara Zigulevsk	AB433845	AB433852
	Samara_68/ CG/2005	*M. glareolus*	Russia/Samara city	AB433844	AB433851
	Samara_94/ CG/2005	*M. glareolus*	Russia/Samara city	AB433847	AB433854
	Samara_147/CG/2005	*M. glareolus*	Russia/Samara city	AB433848	AB433855
	F-s808	HFRS patient	Russia/Samara city	AF411446	

Table 5. Comparison of nucleotide (nt 85 to 1238) and amino acid (aa 15 to 403) sequences of the S segment among hantaviruses

	Identities of nucleotide and amino acid (%)[a]											
	Puumala										Dobrava	Hantaan
	Zigulevsk[b]	Sam68[c]	Sam94[d]	Sam147[e]	F-s808	Kazan	CG1820	Ufa97	Sotkamo	Umea/hu		76–118
Zigulevsk	–	96.5	96.7	96	99.3[f]	95.1	94.2	94.3	85.5	84.5	62.8	62.2
Sam68	99.7	–	98.7	99.5	96.7	94.8	93.5	93.6	85.5	84.6	62.6	62.8
Sam94	99.3	99.2	–	98.2	96.7	94.9	93.5	93.6	85.6	84.4	63.4	62.3
Sam147	98.7	99	98.2	–	96.2	95.2	94.1	94.2	85.2	84.2	62.6	62.9
F-s808	100	99.7	99.3	98.7	–	95.4	94.3	94.4	85.7	84.9	61.8	61.7
Kazan	99.1	98.7	98.4	97.7	99	–	94.3	94.4	85.3	84.2	63	62.4
CG1820	99.1	98.7	98.4	97.7	99	98.6	–	99.8	85.6	84	62.7	62.3
Ufa97	99.3	98.7	98.6	97.7	99.3	98.8	99.3	–	85.6	84.1	62.6	62.2
Sotkamo	96.8	96.7	96.1	96.4	96.8	96.8	96.3	96.5	–	85.5	63.4	62.7
Umea/hu	96.3	96.4	95.6	95.9	96.5	96.3	95.8	96.1	96.5	–	63.2	61.3
Dobrava	61	61.6	60.3	60.6	58.6	60.5	60.5	60.5	61.2	60.7	–	74
Hantaan	61.4	60.2	60.7	59.6	59.3	61.2	61	61	60.7	60.7	83	–

a) Values to the right above the diagonal show nucleotide identities; those to the left below the diagonal show amino acid identities.
b) Zigulevsk; Samara_6/CG/2005, Samara_49/CG/2005, Samara_112/CG/2005 and Samara_128/CG/2005 (the same sequence).
c) Samara_68/CG/2005.
d) Samara_94/CG/2005.
e) Samara_147/CG/2005.
f) Values in bold show the identities between the F-s808 and PUUVs detected from *M. glareolus* in Zigulevsk National Park

(Table 6). However, no PUUV genes were detected in the sera of HFRS patients by RT-PCR (Table 6). ELISA was also used to perform serological analysis using baculovirus-expressed NPs of HTNV, DOBV, and PUUV. All IFA-positive sera had at least two times higher IgG-ELISA values to PUUV than to DOBV or HTNV (Fig. 3A). IgM-ELISA values of the patients also showed the same reactive patterns as IgG-ELISA values (Fig. 3B).

DISCUSSION

The purpose of this study was to investigate the characteristics and ecology of hantaviruses in European Russia. There is an urgent need for clear information concerning the circulation and maintenance of hantaviruses in nature, their association with specific rodent reservoir hosts, and their contribution to HFRS morbidity. The majority of HFRS cases in European Russia are caused by PUUV, with only rare cases caused by DOBV. Human epidemics are charac-

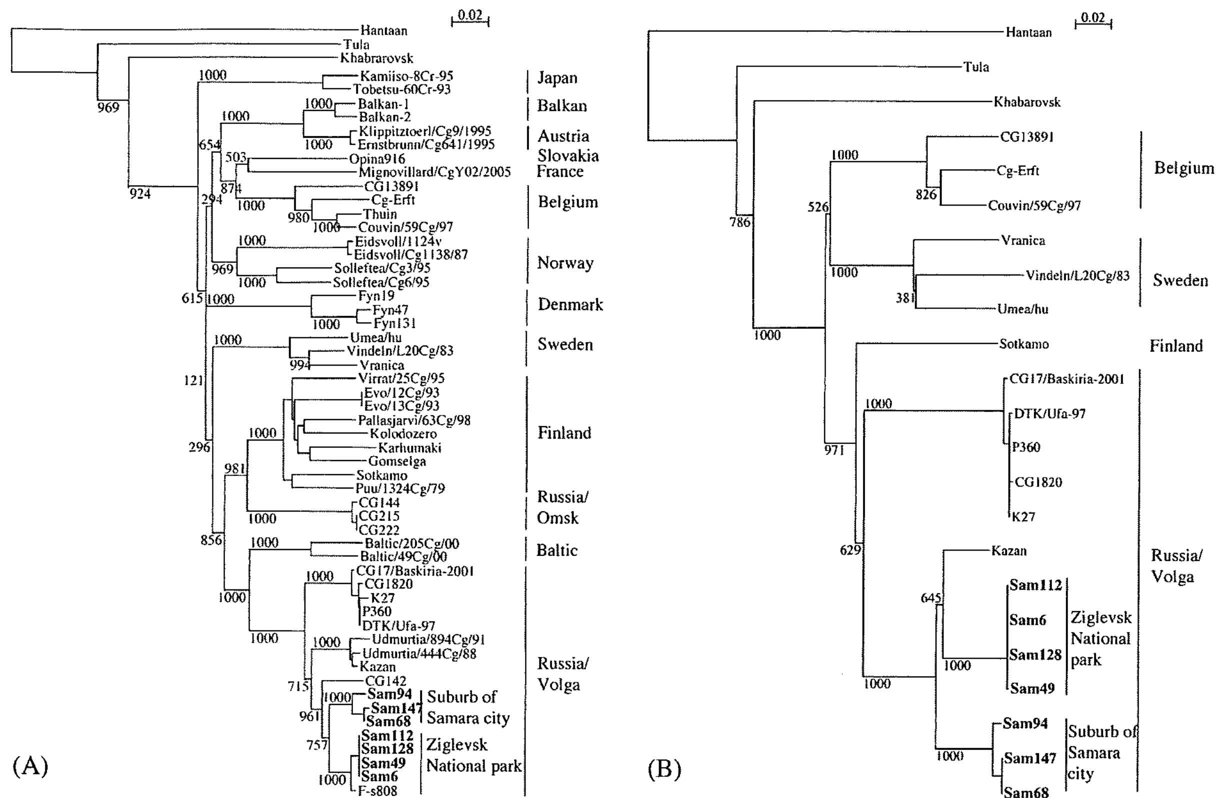

Fig. 2. Hantavirus phylogenetic trees. Hantavirus sequences were aligned using Clustal X, and the phylogenetic trees were generated using the neighbor-joining method. The reliability of the dendrogram was evaluated by 1,000 bootstrap replicates. (A) Phylogenetic tree of hantavirus partial S-segments (nts 85 to 1238). (B) Phylogenetic tree of hantavirus partial M-segments (nts 2484 to 3494).

terized by outbreaks with a frequency of three to four years. The primary determinative factor that influences HFRS epidemics in humans is the periodical and massive reproduction of rodents, together with the development of epizootics in their expanding populations [40].

The results of our study show that PUUV is the major causative agent of HFRS in the Samara region and that *M. glareolus* plays an important role as the reservoir animal in this area. In addition, anti-hantavirus antibodies were detected in one *A. flavicollis* with low titers to HTNV and SEOV but not to PUUV. However, no viral genes were detected in *A. flavicollis*. Further investigation is needed to determine whether DOBV or DOB-related viruses circulate among the *A. flavicollis* population in the Samara region.

Although serum samples were limited, the serological analyses of HFRS patients suggested that PUUV is the major cause of HFRS cases in the Samara region. This observation strongly supports previous findings [21, 25, 43]. The virus sequences of Sam6, Sam49, Sam112, and Sam128 identified in *M. glareolus* at Zigulevsk National Park were almost identical to PUUV from a fatal case of HFRS that occurred in the Samara region. Therefore, it is highly possible that *M. glareolus* in Samara carries PUUV, which causes severe HFRS. Further characterization of the viruses in the Samara region is required to elucidate the pathogenicity of PUUV.

Nucleotide sequence comparisons revealed that the viruses in the Samara region are similar to the PUUV strains Kazan, CG1820, and DTK/Ufa-97 that were isolated near the Samara region within the Volga River area. In addition, phylogenetic analysis clearly demonstrated the presence of PUUVs in the Samara region; viruses originating from the Volga River area occupied one cluster and were distant from European PUUVs. Viruses of Russian origin formed several separate lineages such as those found in Omsk and in the Volga River area. These findings provide insight towards understanding the evolution of PUUV [4, 22, 37].

PUUV is the most common cause of HFRS in Europe and is harbored by the bank vole (*M. glareolus*), which is found in most of Europe. Genetic analyses of strains from the Baltic countries, Austria, Slovakia, France, Belgium, Norway, Denmark, Sweden, Finland, Germany, and Russia have shown PUUV to be the most variable of the hantavirus species; genetic diversity at the nucleotide level reaches 20% and 17% for the coding regions of the M- and S-segments, respectively, and reaches even higher values (37% and 30%) for their 3'-noncoding regions [6, 8, 17, 18, 29, 32].

Interestingly, PUUV strains from the Omsk region (west-

Table 6. Detection of anti-hantavirus antibodies in HFRS patients and patients with high fever of unknown etiology

Patient No.	Serum No.	Age	Diagnosis	Days after onset	IFA titer PUUV	SEOV	HTNV	PCR
1	1–1	43	HFRS	6	256	<32	<32	–[a]
	1–2			20	512	<32	<32	ND[b]
2	2–1	58	HFRS	14	1024	<32	<32	–
	2–2			19	2048≤	<32	<32	ND
3	3–1	44	HFRS	6	512	<32	<32	–
	3–2			26	2048≤	<32	<32	ND
4	4–1	16	HFRS	7	256	<32	<32	–
	4–2			10	256	<32	<32	ND
5	5–1	18	HFRS	1	<32	<32	<32	–
	5–2			6	<32	<32	<32	ND
6	6–1	29	HFRS	10	1024	<32	<32	–
7	7–1	38	HFRS	8	2048≤	<32	<32	–
8	8–1	44	Unknown	4	<32	<32	<32	–
	8–2			12	<32	<32	<32	ND
9	9–1	21	Unknown	5	<32	<32	<32	–
	9–2			8	<32	<32	<32	ND
10	10–1	52	Unknown	8	<32	<32	<32	–
	10–2			11	<32	<32	<32	ND
11	11–1	28	Unknown	7	256	<32	<32	–
12	12–1	28	Unknown	Unknown	<32	<32	<32	–
13	13–1	35	Unknown	5	<32	<32	<32	–

a) –: negative.
b) ND: not done

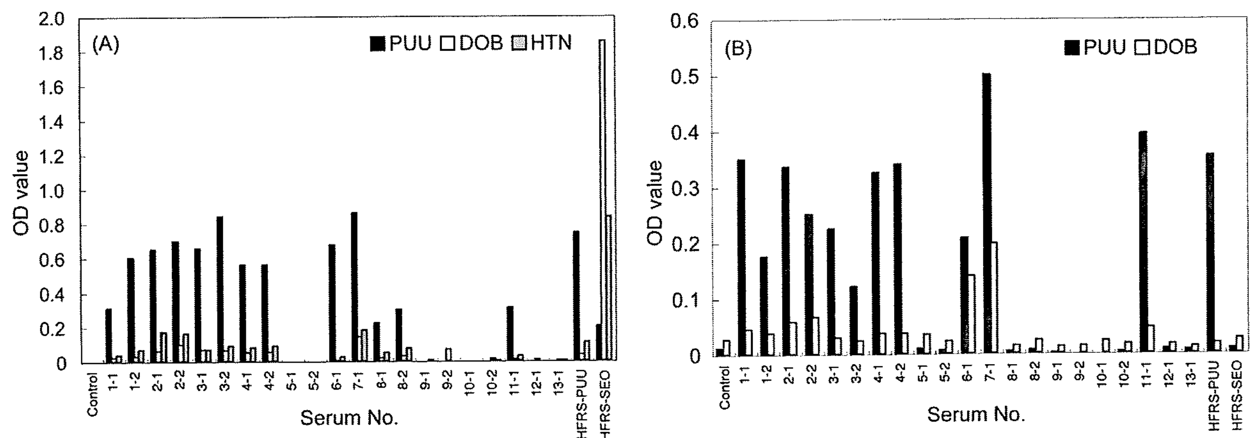

Fig. 3. Detection of anti-hantavirus antibodies in HFRS patients and patients with high fevers of unknown etiologies by ELISA. Baculovirus-expressed hantavirus nucleocapsid proteins (NPs) were used as the antigens. PUU: full length NP of PUUV; DOB: full length NP of DOBV; HTN: full length NP of HTNV; Control: Normal human serum; HFRS-PUU: PUUV-infected HFRS patient serum; HFRS-SEO: SEOV-infected HFRS patient serum. (A) Detection of anti-hantavirus IgG. (B) Detection of anti-hantavirus IgM.

ern Siberia) are more closely related to Finish strains than to the Russian strains isolated from geographically closer regions (e.g., Ufa, Kazan, Udmurtia, or Samara) [4]. Closely related sublineages of PUUV were found in samples obtained from near and distant geographical locations of Russia [4, 7, 23, 24,]. According to a theory proposed by

Plyusnin *et al.* [30,31], the extent of hantavirus diversity is proportional to the geographic distance between the areas of their circulation. However, while the microgeographical relationships among hantaviruses might be linearly proportional, the macrogeographical relationships are likely to be more complex. The macrogeographical evolution of hantaviruses might be significantly biased by distant migrations of animals, especially ones that occurred during the several interglacial epochs [3, 4].

The results obtained in this study further characterize the public health threat caused by PUUV infection in the high HFRS epidemic area of European Russia. The information obtained in this study is quite valuable to prevent HFRS by avoiding contacts to *M. glareolus* and is also quite important to understand the significance of the vaccine development to PUUV infections in European Russia.

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REFERENCES

1. Alexeyev, O. A., Elgh, F., Zhestkov, A. V., Wadell, G. and Juto, P. 1996. Hantaan and Puumala virus antibodies in blood donors in Samara, an HFRS-endemic region in European Russia. *Lancet* **347**: 1483.
2. Araki, K., Yoshimatsu, K., Ogino, M., Ebihara, H., Lundkvist A., Kariwa, H., Takashima, I. and Arikawa, J. 2001. Truncated hantavirus nucleocapsid proteins for serotyping Hantaan, Seoul, and Dobrava hantavirus infections. *J. Clin. Microbiol.* **39**: 2397–2404.
3. Asikainen, K., Hanninen, T., Henttonen, H., Niemimaa, J.,Laakkonen, J., Andersen, H. K., Bille, N., Leirs, H., Vaheri, A. and Plyusnin, A. 2000. Molecular evolution of puumala hantavirus in Fennoscandia: Phylogenetic analysis of strains from two recolonization routes, Karelia and Denmark. *J. Gen. Virol.* **81**: 2833–2841.
4. Dekonenko, A., Yakimenko, V., Ivanov, A., Morozov, V., Nikitin, P., Khasanova, S., Dzagurova, T., Tkachenko, E. and Schmaljohn, C.2003. Genetic similarity of Puumala viruses found in Finland and western Siberia and of the mitochondrial DNA of their rodent hosts suggests a common evolutionary origin. *Infect. Genet. Evol.* **3**: 245–257.
5. Dzagurova, T. K., Tkachenko, E. A., Iunicheva, IuV., Morozov, V. G., Briukhanov, A. F., Bashkirtsev, V. N., Sedova, N. S., Klempa, B. and Kruger, D. 2008. Discovery, clinical and etiological characteristic of HFRS in subtropical zone of Krasnodar region (in Russian). *Zh. Mikrobiol. Epidemiol. Immunobiol.* **1**: 12–16.
6. Hörling, J., Lundkvist, A., Jaarola, M., Plyusnin, A., Tegelström, H., Persson, K., Lehväslaiho, H., Hörnfeldt, B., Vaheri, A. and Niklasson, B. 1996. Distribution and genetic heterogeneity of Puumala virus in Sweden. *J. Gen. Virol.* **77**: 2555–2562.
7. Ivanov, A., Dekonenko., A, Dzagurova,, T., Tkachenko, E., Ivanov, A. P., Dekonenko, A. E, Dzagurova, T. K., Malkin, A. E., Shervarli, V. I, Baranovskiĭ, P. M., Vardanian, N. V., Bulanov, V. E., Nikolaeva, E. I. and Tkachenko, E.A. 2000. Outbreak of HFRS in Egor'evsk district of Moscow region. (in Russian). *Vopr. Virusol.* **45**: 33–36.
8. Khaiboullina, S. F., St.Jeor, S. C. and Morzunov, S. P. 1997. Genetic variability and natural reassortment of the Puumala hantavirus in Bashkiria, Russia. S:210. p. 155. *In*: Emergence and Re-emergence of Negative Strand Viruses. 10th International Conference on Negative Strand Viruses, Dublin, Ireland.
9. Klempa, B., Schmidt, H. A., Ulrich, R., Kaluz, S., Labuda, M., Meisel, H., Hjelle, B. and Krüger, D. H. 2003. Genetic interaction between distinct Dobrava hantavirus subtypes in Apodemus agrarius and *A. flavicollis* in nature. *J. Virol.* **77**: 804–809.
10. Klempa, B., Stanko, M., Labuda, M., Ulrich, R., Meisel, H. and Krüger, D. H. 2005. Central European Dobrava Hantavirus isolate from a striped field mouse (Apodemus agrarius). *J. Clin. Microbiol.* **43**: 2756–2763.
11. Klempa, B., Tkachenko, E. A., Dzagurova, T. K., Yunicheva, Y. V., Morozov, V. G., Okulova, N. M., Slyusareva, G. P., Smirnov, A. and Kruger, D. H. 2008. Hemorrhagic fever with renal syndrome caused by two distinct lineages of Dobrava hantavirus emerging in Russia. *Emerg. Infect. Dis.* **14**: 617–625.
12. Klingstrom, J., Hardestam, J. and Lundkvist, A. 2006. Dobrava, but not Saaremaa, hantavirus is lethal and induces nitric oxide production in suckling mice. *Microbes Infect.* **8**: 728–737.
13. Kruger, D. H., Ulrich, R. and Lundkvist, A. 2001. Hantavirus infections and their prevention. *Microbes Infect.* **3**: 1129–1144.
14. Lee, H.W., Lee, H. W., Baek, L. J. and Johnson, K. M.1982. Isolation of Hantaan virus, the etiologic agent of Korean hemorrhagic fever, from wild urban rats. *J. Infect. Dis.* **146**: 638–644.
15. Leshchinskaia, E. V., Tkachenko, E. A., Ryl'tseva, E. V., Petrov, V. A., Ianovskiĭ, S. M., Gasanova, T. A., Bystrovskiĭ, V. F. , Mogila, T. V., Koval'skiĭ, G. S., Priven, V. Z., *et al.* 1990. Characteristics of endemic foci of hemorrhagic fever with renal syndrome in various regions of the USSR (in Russian). *Vopr. Virusol.* **35**: 42–45.
16. Lokugamage, K., Kariwa, H., Hayasaka, D., Cui, B. Z., 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. 2002. Genetic characterization of hantaviruses transmitted by the Korean field mouse (Apodemus peninsulae), Far East Russia. *Emerg. Infect. Dis.* **8**: 768–776.
17. Lundkvist, A., Cheng, Y., Sjölander, K. B., Niklasson, B., Vaheri, A. and Plyusnin, A. 1997. Cell culture adaptation of Puumala hantavirus changes the infectivity for its natural reservoir, Clethrionomys glareolus, and leads to accumulation of mutants with altered genomic RNA S segment. *J. Virol.* **71**: 9515–9523.
18. Lundkvist, A., Wiger, D., Hörling, J., Sjölander, K. B., Plyusnina, A., Mehl, R., Vaheri, A. and Plyusnin, A. 1998. Isolation and characterization of Puumala hantavirus from Norway: evidence for a distinct phylogenetic sublineage. *J. Gen. Virol.* **78**: 2603–2614.
19. Miyamoto, H., Kariwa, H., Araki, K., Lokugamage, K.,

Hayasaka, D., Cui, B. Z., Lokugamage, N., Ivanov, L. I,, Mizutani, T., Iwasa, M. A,, Yoshimatsu, K., Arikawa, J. and Takashima, I. 2003. Serological analysis of hemorrhagic fever with renal syndrome (HFRS) patients in Far eastern Russia and identification of the causative hantavirus genotype. *Arch. Virol.* **148**: 1543–1556.

20. Morii, M., Yoshimatsu, K., Arikawa, J., Zhou, G., Kariwa, H. and Takashima, I. 1998. Antigenic characterization of Hantaan and Seoul virus nucleocapsid proteins expressed by recombinant baculovirus: application of a truncated protein, lacking an antigenic region common to the two viruses, as a serotyping antigen. *J. Clin. Microbiol.* **36**: 2514–2521.
21. Morozov, V., Korobov, G. and Tkachenko, E. 2002. Epidemiological and molecular-genetical characteristics of HFRS in Samara region. (in Russian) Kazan *Med. J. v.LXXXIII*; **2**: 151–155.
22. Morozov, V., Morzunov, S., Khaiboullina, S., Dzagurova, T. *et al.* 2004. Genetic typing of hantaviruses in HFRS patient blood sera. (in Russian) *Epidemiology and Infect. Dis.* **2**: 43–47.
23. Morzunov, S. *et al.* 1999. p. 295. Proceedings of the Abstracts of the XI International Congress of Virology. Sydney, Australia.
24. Nikonorov, Yu, Khasanova, S,Tkachenko, E. *et al.* 2001. Analysis of primaryRNA from HFRS in the Republic of Bashkortostan. (in Russian). Molecular Genetica, *Microbiol. Virol. J.* **1**: 35–39.
25. Nurgaleeva, R.G., Tkachenko, E.A., Stepanenko, A.G., Mustafin, I. *et al.* 1999. An epidemiological analysis of hemorrhagic fever with renal syndrome morbidity in the Republic of Bashkortostan in 1997. (in Russian) *J. Mikrobiol. Epidemiol. Immunobiol.* **12**: 45–49.
26. Okumura, M., Yoshimatsu, K., Araki, K., Lee, B. H., Asano, A., Agui, T. and Arikawa, J. 2004. Epitope analysis of monoclonal antibody E5/G6, which binds to a linear epitope in the nucleocapsid protein of hantaviruses. *Arch. Virol.* **149**: 2427–2434.
27. Okumura, M., Yoshimatsu, K., Kumperasart, S., Nakamura, I., Ogino, M., Taruishi, M., Sungdee, A., Pattamadilok, S., Ibrahim. I. N., Erlina, S., Agui, T., Yanagihara, R. and Arikawa, J. 2007. Development of serological assays for Thottapalayam virus, an insectivore-borne Hantavirus. *Clin. Vaccine Immunol.* **14**: 173–181.
28. Padula, P. J., Colavecchia, S. B., Martínez, V. P., Gonzalez Della Valle, M. O., Edelstein, A., Miguel, S. D., Russi, J., Riquelme, J. M., Colucci, N., Almirón, M. and Rabinovich, R. D. 2000. Genetic diversity, distribution, and serological features of hantavirus infection in five countries in South America. *J. Clin. Microbiol.* **38**: 323–330.
29. Plyusnin, A, Vapalahti, O, Lankinen, H, Lehväslaiho, H., Apekina, N., Myasnikov., Kallio-Kokko, H., Henttonen, H., Lundkvist, A., Bummer-Korvenkontio, M., Gavrilovskaya, I. and Vaheri, A. 1994. Tula virus - a newly detected hantavirus carried by European common voles. *J. Virol.* **68**: 7833–7839.
30. Plyusnin, A., Vapalahti, O., Lehväslaiho, H., Apekina, N., Mikhailova, T., Gavrilovskaya, I., Laakkonen, J., Niemimaa, J., Henttonen, H., Brummer-Korvenkontio, M. and Vaheri, A. 1995. Genetic variation of wild Puumala viruses within the serotype, local rodent populations and individual animal. *Virus Res.* **38**: 25–41.
31. Plyusnin, A., Vapalahti, O., Ulfves, K., Lehväslaiho, H., Apekina, N., Gavrilovskaya, I., Blinov, V. and Vaheri, A. 1994. Sequences of wild Puumala virus genes show a correlation of genetic variation with geographic origin of the strains. *J. Gen. Virol.* **75**: 405–409.
32. Plyusnin, A. and Morzunov, S.P. 2000. Virus evolution and genetic diversity of hantaviruses and their rodent hosts. pp. 47–76. *In*: Hantaviruses. Current Topics in Microbiology and Immunology (Nichol, S.T. and Schmaljohn, C.S. eds.), Springer-Verlag, Berlin - New York.
33. Schmaljohn, C. and Hjelle, B.1997. Hantaviruses: a global disease problem. *Emerg. Infect. Dis.* **3**: 95–104.
34. Schmaljohn, C. 2001. p. 256. *In*: Hantaviruses. Current Topics in Microbiology and Immunology (Nichol, S.T. ed), Berlin: Springer-Verlag.
35. Schmidt Chanasit, J., Essbauer, S.S., Petraityte, R., Yoshimatsu, K, *et al.* 2007. p. 38. Longitudinal study in Germany: occurrence of Tula virus in Microtus arvalis and Microtus agrestis. Abstr. VII International conference on HFRS, HPS and hantaviruses, Buenos Aires, Argentina.
36. Sibold, C., Sparr, S., Schulz, A., Labuda, M., Kozuch, O., Lysý, J., Krüger, D. H. and Meisel, H. 1995. Genetic characterization of a new hantavirus detected in Microtus arvalis from Slovakia. *Virus Genes.* **10**: 277–281.
37. Sironen, T., Vaheri, A. and Plyusnin, A. 2001. Molecular evolution of Puumala hantavirus. *J. Virol.* **75**: 11803–11810.
38. Slonova, R. A., Tkachenko, E. A. and Astachova, T. I. 1990. Hantavirus serotypes circulating in foci of the Far East region of the USSR. (in Russian) *Vopr. Virusol.* **5**: 391–393.
39. Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **24**: 4876–4882.
40. Tkachenko, E., Dekonenko, A., Ivanov, A., Dzagurova, T. *et al.* 1999. Hemorrhagic fever with renal syndrome and hantaviruses in Russia. pp. 63–72. *In*: Dodet B, ed. Emergence and Control of Rodent-borne Viral Diseases, Elsevier.
41. Tkachenko, E., Dzagurova, T., Bashkirtsev, V., Bernshtein, A. *et al.* 2007. p. 17. Epidemiology of HFRS in European Russia. Abstract book. VII Intern. Conf. on HFRS, HPS and Hantaviruses. Buenos Aires, Argentina.
42. Tkachenko, E. A., Okulova, N. M., Yunicheva, YuV., Morzunov, S. P., Khaĭbulina, S. F., Riabova, T. E., Vasilenko, L. E., Bashkirtsev, V. N., Dzagurova, T. K., Gorbachkova, E. A., Sedova, N. S., Balakirev, A. E., Dekonenko, A. E. and Drozdov, S. G.. 2005. The epizootiological and virological characteristics of a natural hantavirus infection focus in the subtropical zone of Krasnodarsk Territory. *Vopr. Virusol.* **50**: 14–19.
43. Vapalahti, O., Mustonen, J., Lundkvist, A., Henttonen, H., Plyusnin, A. and Vaheri, A. 2003. Hantavirus infections in Europe. *Lancet Infect. Dis.* **3**: 653–661.
44. World Health Organization. 1983. Haemorrhagic fever with renal syndrome. *Bull. WHO.* **61**: 269–275.
45. Yakimenko, V, Dekonenko, A, Malkova, M, Kuzmin, I, *et al.* 2001. p. 164. Natural foci of hantaviruses in West Siberia. Abstr. of 5th Intern. Conference on HFRS, HPS, and hantaviruses. France, Veyrier-du-Lac.
46. 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. 2000. Genetic diversity of hantaviruses associated with HFRS in far east of Russia. *Virus Res.* **70**: 31–44.
47. Yoshimatsu, K., Arikawa, J., Tamura, M., Yoshida, R., Lundkvist, A., Niklasson, B., Kariwa, H. and Azuma, I. 1996. Characterization of the nucleocapsid protein of Hantaan virus strain 76–118 using monoclonal antibodies. *J. Gen. Virol.* **77**: 695–704.

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Acute febrile illness caused by hantavirus: serological and molecular evidence from India

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Summary Study of hantavirus infections in India is in its early stages. As early symptoms of hantavirus disease can be non-specific and the diagnosis confirmed only by laboratory testing, use of appropriate diagnostic tools is important. To improve the diagnosis of hantavirus infections in India, commercial ELISA systems followed by indirect immunofluorescence assays were used to detect anti-hantavirus IgM and IgG in samples from patients with acute febrile illness. Of 347 patients tested, 5.2% showed serological evidence of hantavirus infection. Sequences obtained from patients showing molecular evidence of hantavirus infection were related to Hantaan virus. In the absence of μ-capture ELISA, we recommend the use of combination testing systems in areas non-endemic for hantavirus infections. In India there is an increased risk of rodent-borne infections and the differential diagnosis of undifferentiated febrile illness should include hantavirus infection.
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1. Introduction

Hantaviruses belong to the family Bunyaviridae and cause two important clinical syndromes, haemorrhagic fever with renal syndrome (HFRS) in Europe and Asia and hantavirus cardiopulmonary syndrome (HCPS) in the Americas.[1] Worldwide there are approximately 100 000 annual cases of HFRS. Since 1993, HCPS cases have been reported regularly from the Americas.[2]

Hantaviruses are negative-stranded RNA enveloped viruses with a tripartite genome. These rodent-borne viruses (roboviruses) are transmitted to humans through inhalation of aerosols from urine, faeces and saliva of infected rodents.[3] The medically important species causing HFRS are Hantaan virus (HTNV), Seoul virus (SEOV), Puumala virus

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(PUUV) and Dobrava-Belgrade virus (DOBV), whilst Sin Nombre virus (SNV) causes HCPS.[4,5]

Thottapalayam virus (TPMV), named after an area in Vellore town in south India, is the only hantavirus isolated from India from the spleen of an insectivore, *Suncus murinus*, in 1964.[6] It is a poorly studied serotype and its pathogenicity in humans is not well understood. It is genetically and antigenically very diverse from other hantaviruses.

Clinically, the febrile illnesses produced by dengue virus, *Leptospira* and hantavirus are often indistinguishable.[7] The severity of human hantavirus infection depends on the infecting serotype. During evaluation of HFRS patients, physicians may commonly encounter three kinds of clinical manifestations: shock with multiorgan failure; acute renal disease; and acute undifferentiated febrile illness without renal failure.[8]

It is difficult to diagnose HFRS clinically as the initial symptoms can be non-specific. Laboratory diagnosis of HFRS infections relies on serology, as the viraemia in HFRS is short lived.[9] The N protein of hantaviruses is the immunodominant antigen employed for serodiagnosis.

ELISA is more sensitive than immunofluorescence assay (IFA); however, IFA, when used in tandem with an ELISA system, improves the overall specificity. Baculovirus-derived antigens employed in ELISA systems are antigenically similar to native protein.[10] ELISA systems using a cocktail of antigens have a lower sensitivity and specificity compared with systems using homologous antigens but are useful in areas where pilot epidemiological studies are being conducted, where the circulating serotype(s) is unknown and where multiple hantaviruses may be co-circulating.[11]

The S segment that codes for the N protein is generally conserved and primers from this segment are used in molecular diagnosis.[12–15] The time of sample collection and the primers used are important factors influencing the sensitivity of RT-PCR.[16] Region-specific primers can increase the sensitivity of nested RT-PCR.[15] However, in areas where circulating serotypes are not known, broad-based genus-specific primers are the choice for RT-PCR.

Increased hantavirus cases coincide with the increase in agricultural activities during spring and fall and the breeding cycles of rodent hosts. Large outbreaks are usually associated with an increased rodent population.[14]

Studies on hantavirus infections in India are still in the early stages. Here we describe the use of serological and molecular techniques to investigate acute febrile illness caused by hantavirus.

2. Materials and methods

This study was a prospective, observational study conducted on patients admitted to the medical wards of Christian Medical College, Vellore, India, during the period 2005–2007. The inclusion criterion was a history of acute undifferentiated febrile illness (temperature $\geq$101 °F) of 3 days to 2 weeks duration. All study patients gave informed consent.

Blood samples ($n = 347$) were collected in EDTA for hantavirus testing by serological and molecular methods. Paired samples (acute and convalescent) were obtained from 37 patients. Samples were processed immediately on receipt and the plasma and buffy coat samples were stored at −70 °C until testing.

All samples were tested for the presence of hantavirus IgM by a commercial ELISA (Hantavirus Dx Select; Focus Diagnostics, Cypress, CA, USA) and seropositives were further tested by an IFA.[17] All IgM positives were also further tested for hantavirus IgG by a screening IgG ELISA (Focus Diagnostics) followed by IgG IFA.[18] According to the manufacturer, the commercial kits can detect antibodies against HTNV, SEOV, PUUV and SNV. Positive and negative controls for the ELISA systems were provided with the kits. For the ELISA systems the cut-off index value [optical density (OD) of sample/OD of calibrator] was >1.1. For IFA, a grading $\geq$2 was considered positive. The positive control for the IFA was a sample with high anti-SEOV IgM titre. Negative controls were non-reactive for anti-hantavirus IgM and IgG antibodies by ELISA and IFA.

Testing for both anti-hantavirus IgM and IgG in patients with acute febrile illness was done to improve the diagnosis of hantavirus infections.

Of the 347 patient samples, 266 samples were tested for the presence of hantavirus RNA by a conventional in-house RT-PCR.

Fifty cord blood samples were tested to check the specificity of the serological assays used in the study, i.e. commercial IgM ELISA and IgM IFA. The cord blood samples were received from the Department of Obstetrics and Gynecology at Christian Medical College.

Serological evidence of hantavirus infection was defined as follows: single acute sample when tested should be positive for anti-hantavirus IgM (by ELISA and IFA) and IgG (by ELISA and IFA), and paired samples (acute and convalescent) should show a significant difference in IgG titre between acute phase and convalescent phase samples or show seroconversion. Molecular evidence of hantavirus infection was the demonstration of hantavirus RNA in clinical samples.

Total RNA was extracted from buffy coat samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was done using Moloney murine leukemia virus reverse transcriptase enzyme (Invitrogen Corp., Carlsbad, CA, USA) and random hexamers. Amplification was performed using a nested PCR protocol with primers specific for the 973–1253 nucleotide region of the S segment. Precautions were taken to reduce contamination during molecular testing. The areas for extraction, amplification and detection are physically separate and have dedicated equipment, and unidirectional workflow is always maintained. Only one tube was opened at a time. Water controls (no template) were used after every two samples. Filter-blocked pipette tips were used in all steps. Disposable gloves were changed between samples. Biosafety cabinets were used for specimen processing and extraction and for preparing the PCR reagent mix. Before and after use, the cabinets were cleaned and irradiated with UV light. The positive control used was HTNV cDNA provided by Dr Connie Schmaljohn [United States Army Medical Research Institute of Infectious Diseases (USAMRIID), Fort Detrick, MD, USA]. The first round primers were 5′-GAT AGG TGT CCA CCA ACA TG-3′ and 5′-AGC TCT GGA TCC ATG TCA TC-3′, whilst the second round primers were 5′-GCA GGT ATT GCT GAG CTT GG-3′ and 5′-TCC CCA TTG ATT GTG TCC TT-3′. Supratherm *Taq* DNA Polymerase (GeneCraft, Lüdinghausen, Germany) was

used for PCR. After the second round of amplification, a 154 bp product was obtained. Both the primer sets were able to amplify cDNA of HTNV and SEOV. The specificity of the primers was ascertained by testing RNA extracts of measles virus, poliovirus, Japanese encephalitis virus and hepatitis C virus. The integrity of the extraction protocol was verified by amplifying β-actin RNA in all the samples. The second round products were subjected to sequencing and the nucleotide sequences obtained were subjected to BLAST to identify the sequences obtained. Nucleotide sequences from GenBank of the corresponding amplified region of HTNV (D25530), SEOV (M34881), Thailand virus (THAIV) (AB186420) and TPMV (AY526097) were used to construct a phylogenetic tree using the ClustalW alignment algorithm of the MegAlign program version 5.05 (DNASTAR Inc., Madison, WI, USA).

3. Results

Overall, 24 patients showed serological or/and molecular evidence of hantavirus infection. Serological tests were conducted on 347 patients, whilst only 266 of those were tested by RT-PCR. Serological evidence of hantavirus infection as per our criteria mentioned in the Materials and Methods was evidenced in 18 patients (5.2%). Six patients (2.3%) showed molecular evidence of hantavirus infection.

Anti-hantavirus IgM was detected in 78 (22.5%) of the patients. However, only 18 of these fulfilled the accepted serological criteria for hantavirus infection.

Fifty cord blood samples were negative for anti-hantavirus IgM with the two assays used (ELISA and IFA). Comparison of the fraction of positive cord blood samples with the fraction of positive patient samples was not statistically significant, which could be attributed to the small number of cord blood samples tested.

Table 1 presents an overall picture of the reactivity of each patient's sample in individual serological tests. The age range of the patients who presented with evidence of hantavirus infection in this study was 12–70 years and included 13 males and 11 females. Hantavirus-specific IgM could be detected as early as 3 days after the onset of illness. The mean IgM ELISA index value of samples collected 3–4 days, 5–9 days and ≥10 days after the onset of illness was 3 (±1.97), 3.2 (±2.7) and 2.4 (±1.2), respectively. The mean IgM ELISA index of all reactive samples was 2.8 (±1.98). Two of the six patients positive for hantavirus RNA (#19 and #22) did not show the presence of hantavirus-specific IgM antibodies, and in one sample (#24) HTNV IgM IFA was negative.

Supplementary Figure 1 shows standardisation of first and second round PCR with HTNV cDNA, second round products from a patient's sample, nested PCR results with SEOV cDNA and a representative gel picture of β-actin from patient samples.

Table 1 Serology of patients ($n=24$) with evidence of hantavirus (HTNV) infection

Sample ID[a]	Age (years)	Sex	Time post onset (days)	HTNV IgM ELISA	HTNV IgM IFA	HTNV IgG ELISA	HTNV IgG IFA	Leptospirosis	Dengue
#1	65	M	10	3.4	>2+	1.2	4+	N	NT
#2	20	F	3	4.4	>2+	10	3+	N	N
#3	41	F	8	3.2	>2+	1.7	>2+	NT	P
#4	47	F	7	9.2	3+	4.3	3+	NT	P
#5	64	M	10	2.5	3+	1.3	2+	P	NT
#6	25	F	7	1.7	2+	1.98	>2+	N	NT
#7	40	F	10	2.3	>2+	1.9	3+	N	NT
#8	23	F	7	1.2	2+	1.14	2+	N	N
#9	42	F	10	2.2	2+	1.4	>2+	N	NT
#10	40	M	10	2	4+	1.25	2+	N	N
#11	45	M	10	5.6	>2+	1.7	2+	N	NT
#12	60	F	10	2.1	3+	HP	3+	N	NT
#13	35	F	5	5.6	2+	1.2	>2+	N	NT
#14	32	M	8	4.4	3+	1.6	3+	NT	NT
#15	30	F	10	1.3	3+	1.7	3+	N	NT
#16	34	M	10	3	2+	1.3	<3+	N	N
#17 Acute	30	M	7	3.8	3+	0.5	N	P	NT
#17 Convalescent				1.8	2+	1.14	2+		
#18 Acute	46	M	5	1.12	2+	N	N	N	N
#18 Convalescent				1.7	2+	1.3	3+		
#19	21	M	15	N	NT	NT	NT	NT	NT
#20,1SS	12	M	4	1.6	2+	N	N	N	N
#21,2SS	57	M	10	1.6	2+	N	N	N	NT
#22,3SS	20	F	10	N	NT	NT	NT	N	NT
#23,4SS	40	M	7	1.4	3+	N	2+	P	NT
#24,5SS	70	M	10	1.2	N	N	2+	N	N

IFA: immunofluorescence assay; N: negative; P: positive; NT: not tested; HP: high positive; SS: identification of sequences from sample.
[a] #1 to #16: single acute phase samples; #17 and #18: paired acute and convalescent samples; #19 to #24: hantavirus RNA positives.