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Genetic diversity of hantaviruses associated with hemorrhagic fever with renal syndrome in the far east of Russia

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Abstract

To identify the hantaviruses causing hemorrhagic fever with renal syndrome (HFRS) in the Far East of Russia, blood samples collected from HFRS patients in 1994–1998, were examined by reverse transcription–polymerase chain reaction. In addition, 36 sera were tested by an immunofluorescence assay for antibodies against Hantaan, Seoul, Puumala, and Khabarovsk viruses, and 54 samples were tested by plaque reduction neutralization test. With both serological assays, the highest antibody titers were to Hantaan and/or Seoul viruses. Of 110 blood samples 36 were found RT-PCR positive. Phylogenetic analysis the sequences of a 256-nucleotide (nt) fragment of the hantavirus M genome segment revealed at least 3 genetically distinct hantavirus lineages. Nucleotide sequence comparison showed that two of the lineages, designated as FE and Amur (AMR), differed from one another by 15.9–21.2% and from Hantaan virus by 9.8–17.5%. The third lineage, VDV, differed from Seoul virus by 2.6–5.1%. All S segment sequences were from FE lineage, and differed from Hantaan virus by 10.7–12.6%. Thirty of the 36 (83%) analyzed sequences were found to be the FE genotype, which is very similar to that of Hantaan virus, strain 76-118. Of the remaining hantaviruses, 11% were the AMR genotype, and 6% the VDV genotype, which are genetically novel genotypes of Hantaan or Seoul viruses, respectively. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Hemorrhagic fever with renal syndrome; Hantaan virus; Seoul virus; Amur virus; PCR

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1. Introduction

Hantaviruses, the causative agents of hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome, are serologically related viruses of the family *Bunyaviridae* and have a worldwide distribution. To date, at least 16 distinct hantaviruses have been identified which represent unique serotypes or genotypes (Nichol et al., 1993; Chu et al., 1994; Xiao et al., 1994; Horling et al., 1996; Plyusnin et al., 1996a). Hantaan (HTN), Puumala (PUU), Seoul (SEO) and Dobrava (DOB) viruses cause about 150 000 HFRS cases annually of varying severity in Eurasia (LeDuc, 1989; Lee et al., 1990; Clement et al., 1997). Each of these hantaviruses is predominantly associated with a single rodent species as its primary natural reservoir: HTN virus with the striped field mouse *Apodemus agrarius*, SEO virus with *Rattus norvegicus* and *Rattus rattus*, PUU virus with the bank vole *Clethrionomys glareolus*, and DOB virus with the yellow-necked field mouse *Apodemus flavicollis*. Recently, DOB virus was also detected in *A. agrarius* in Europe (Nemirov et al., 1999; Plyusnin et al., 1999; Scharninghausen et al., 1999).

A mild and moderate form of HFRS is endemic in European Russia and is caused by PUU virus (Xiao et al., 1993; Dzagurova et al., 1995). Clinically severe cases of HFRS are more characteristic in the Far East of Asian Russia. Serological studies suggest that numerous hantaviruses are present in humans and rodents in this area (Astakhova et al., 1990; Ivanov et al., 1990; Slonova et al., 1990a; Dzagurova et al., 1995). A new hantavirus, Khabarovsk (KBR) virus, was isolated from reed voles (*Microtus fortis*) in Far East Russia but it is not at present implicated in human disease (Horling et al., 1996; Dekonenko et al., 1996). Recently, a hantavirus (designated Vladivostok), was identified in *Microtus fortis* in this region (Kariwa et al., 1999). Hantavirus antibodies in humans and hantavirus antigens in various rodent species have been found in Western and Eastern Siberia; however, neither the serotype nor genetic structure of the viruses has been determined (Myasnikov et al., 1992; Malkin et al., 1996; Butenko et al., 1997). Another hantavirus, Topografov (TOP)

virus, was identified recently in *Lemmus* from Arctic Siberia, but no human pathogenicity was recognized (Plyusnin et al., 1996b; Vapalahti et al., 1999).

The purpose of the present study was to determine the serologic and genetic diversity of hantaviruses responsible for HFRS in the Far East of Russia during 1994–1998.

2. Materials and methods

2.1. Blood samples

Blood samples of 110 HFRS patients, collected during 1994–1998 in the Primorsky (50 cases) and Khabarovsk (60 cases) regions were used in the study. The maximal distance between the residences of the patients was 700 km. ‘Major criterion’ for selection of HFRS cases for our study was the availability of blood samples drawn not later than 10 days after onset of disease. From 45 of these 110 patients, two or three sequentially drawn serum samples (with intervals of approximately 7 days) were available. If possible, the later serum samples were used for serological analyses. The patients recorded in 1996 (37%) and 1997 (45%) represented the majority of the selected cases, 13% samples were collected in 1994–1995, and 5% in 1998. The samples collected included 39% from severe HFRS; 54%, moderate; and 7%, mild.

Fifty-four sera from the Primorsky region (1996–1997) were selected independently for plaque-reduction neutralization analysis. Twelve sera were obtained from previously selected patients, as well as sera from 42 additional patients.

Clinical diagnosis of ‘HFRS’ or ‘suspicion of HFRS’ was confirmed by the presence or titer rise in paired sera of antibodies reactive with HTN, SEO or PUU viruses in all patient sera. The specific antibodies were detected by indirect immunofluorescence assay (IFA) using either cell culture antigen of HTN virus, or a polyvalent HFRS diagnostic kit based on HTN, PUU, and SEO viral antigens and produced by Pilot-Commercial Enterprise of the Chumakov Institute of Poliomyelitis and Viral Encephalitis, Russian Academy of Medical Sciences. The samples were

stored at -40°C , although some of them had been thawed and refrozen several times. Five batches of samples were sent by air mail to the State Research Center of Virology and Biotechnology 'Vector' from the Khabarovsk Antiplague Station and the Institute of Epidemiology and Microbiology, Vladivostok.

2.2. *Virus strains*

Strains of HTN virus (76-118) (Lee et al., 1978), SEO virus (SR-11) (Kitamura et al., 1993), PUU virus (CG 18-20) (Tkachenko et al., 1984), and KBR virus (mf-43) (Dzagurova et al., 1995) were used to prepare viral antigens. All viruses were propagated in Vero-E6 cells according to previously published methods (Dzagurova et al., 1988).

2.3. *Immunofluorescent assay (IFA)*

Spot slides of hantavirus-infected Vero-E6 cells were prepared and stained as described previously (Dzagurova et al., 1988). The slides were incubated with a human serum (two-fold dilutions starting from 1:16), and specific antibody binding was detected by fluorescein-labeled rabbit anti-human immunoglobulins IgG and IgM.

2.4. *Plaque reduction neutralization test (PRNT)*

End point titers of neutralizing antibodies in human sera were determined by PRNT on monolayers of Vero-E6 cells according to methods described earlier (Takenaka et al., 1985).

2.5. *Nested reverse transcription (RT)-PCR and sequence analysis*

The blood clots of serology-positive patients in the acute-phase (days 4–10) of the disease were used for RNA isolation. Total RNA was extracted from 100 mg of blood clots using RNeasy Mini Kit according to the manufacturer's instructions (QIAGEN GmbH, Germany).

Amplification products were obtained by reverse transcription and nested PCR. RT-PCR was performed as a 'one tube' reaction using an Access RT-PCR kit (Promega Corp., Madison, WI)

and suitable annealing temperatures, listed in Table 1. DNA products were separated on 2% agarose or 6% polyacrylamide gel, sliced from the gel, and were purified using a QIAquick Gel Ex kit (QIAGEN GmbH, Germany) for agarose gels and electroelution for polyacrylamide gels.

The purified PCR products from M genomic segments were sequenced by a manual dideoxy cycle sequencing technique according to methods described previously (Kretz et al., 1994). Sequences were obtained from both strands of each PCR product.

PCR-amplified products from the S segment were cloned in the pGEM-T vector (Promega Corp., Madison, WI) or pMOS Blue vector (Amersham Life Science, Buckinghamshire, UK) according to the manufacturer's instructions. The nucleotide sequences of at least two clones of each sample were determined by the Maxam–Gilbert method (Maxam and Gilbert, 1977).

2.6. *Oligonucleotide primers*

KBR/PUU/PH-specific primers, constructed earlier (Horling et al., 1996), and a new set of HTN/SEO-specific primers for M segment were used in the study (Table 1). A new nested set of primers specific to the N region of the hantavirus S RNA genome segment of SEO and HTN viruses was constructed. The primers for the S segment, PUU virus- and KBR virus- specific, which were synthesized and used in the first round PCR, were similar to those described earlier (Plyusnin et al., 1994). For the second round PCR, we designed additional primers, presented in Table 1. We were able to sequence from 233 to 286 nucleotides (generally 256 nt) of the 286 nt amplicons from the M segment. For the 356 nt S segment amplicon we were able to obtain from 325 to 356 nucleotides (generally 356 nt) for all samples except # 7732 for which we obtained 208 nt. In the homology analysis, we compared only regions that were common to all samples (M: 2735-2968, S: 605-923). Likewise, for phylogenetic analyses and amino acid comparisons, regions in common for the majority of the samples were used (M: 2725-2981, S: 592-947).

2.7. Phylogenetic analysis

Analysis of nucleotide and deduced amino acid sequence differences and alignments of these sequences were performed using the CLUSTAL W program (Thompson et al., 1994). Phylogenetic analyses of nucleotide sequence differences between the virus-specific PCR products and different hantavirus strains were carried out by the distance-based neighbor-joining method (MEGA, version 1.02) (Kumar et al., 1993). For neighbor-joining, the Tamura model was used; it is based on the estimation of the frequencies of different nucleotides in the analyzed sequences and of the differences in the rate of transitional and transversional substitutions. Bootstrap confidence limits were obtained by 1,000 replicates of the analysis. Viruses used for analysis (GenBank accession numbers are given in parentheses for the M and S segments): HTN virus strains 76-118 (M14627,

M14626), HoJo (D00376), Lee (D00377); SEO virus strains 80-39 (S47716), SR-11 (M34882, M34881), B1 (X53861), KI-83 (D17592), and R22 (S68035); PUU virus strains Sotkamo (X61034, x61035), CG 18-20 (M29979, M32750); PH virus strain PH-1 (X55129, X34011); SN virus strain CC107 (L33474, L33683); KBR virus (U35254, U35255); and DOB virus (L33685, l41916).

2.8. Nucleotide sequence accession numbers

The nucleotide sequences reported in this article can be obtained from GenBank. The accession numbers are AF110773, AF172413-AF172416, AF172418-AF172442 for the fragments of hantavirus M genome segment, and AF175690-AF175693, AF175695-AF175699, AF175701-AF175706 for the fragments of the S genome segment.

Table 1
Oligonucleotide primers for nested RT-PCR used in the study

Virus specificity (genomic region)	Primer	Position ^a , orientation, sequence (5'–3')	# rounds of PCR (annealing temp)
HTN/SEO (M segment, G2)	HS1	2636(+) AC(A/C)TGTC(A/C)TTTGG(A/T)GACCC	RT-PCR (44°C)
	HS2	3072(–) TCACA(G/A)GCCTTTATTGA(G/T)GT	
	HS3	2715(+) T(T/C)AGGAA(GA)AAATG(TC)AACTTTGC	Nested PCR (46°C)
	HS4	3000(–) ACACC(A/T)GAACCCAGGC(A/C)CC	
KBR/PUU/PH (M segment, G2)	PP1	2644(+) ATTTAA(G/A)CAATGGTG(C/T)ACTAC(T/A)AC	RT-PCR (38°C)
	PP2	3117(–) CC(G/A)TAACACATTGC(A/G)GC	
	PP3	2743(+) TAGAAAGAAATGTGCATTTGC	Nested PCR (41°C)
	PP4	3024(–) CCTGA(G/A)CCCCATGC(A/T/C)CCATC	
HTN/SEO (S segment, N)	KPS1	361(+) ATTGATGAACCTACAGGACAGACAGC	RT-PCR (48°C)
	KPS2	1001(–) A(C/T)AAACAA(A/G)CATGTTGGTGGACA	
	KPS3	592(+) TC(A/C)AGCATGAAGGC(A/T)GAAGAGAT	Nested PCR (56°C)
	KPS4	947(–) GATGATGGTGA CTCAATAT(G/T)TTCAA	
PUU/KBR (S segment, N)	P1	1(+) TAGTAGTAGAC	RT (42°C) PCR (54°C)
	P2	1(+ –) TTCTGCAGTAGTAGTAGACTCCTTGAAAAG	
	YSPR	992(–) C(A/T)GGTGCACA(G/T)GCAANACCC	Nested PCR 52
	YSPF	681(+) G(T/A)AACATCATGAG(T/C)CC(A/T)GT(TAG) ATGGG	

^a The numbers correspond to genome positions of HTN virus, strain 76–118 for HTN/SEO serotypes and PUU virus, strain CG 18–20 for PUU/KBR/PH serotypes.

Fig. 1. Seasonal dynamics of the HFRS morbidity in Khabarovsk region during 1996–1997.

3. Results

3.1. Epidemiology of HFRS in 1996–1997 in the Khabarovsk region

In 1996 and 1997, 41 and 76 HFRS patients, respectively, were laboratory-diagnosed in the territory of Khabarovsk region, 1 and 3 of them with a fatal outcome, respectively. The HFRS cases recorded in 1996 and 1997 were located within the traditional foci of hantavirus infection. As previously, the majority of the cases were recorded in the southern part of the region (valleys of Ussuri and Amur Rivers). Contributing factors to the endemnicity of HFRS in these regions include the predominance of a meadow-field biotic community with the striped field mouse *A. agrarius* as a dominant species, and a higher village population density in these areas, which results in frequent contact of humans with rodents.

Annual dynamics of morbidity over the 2-year period showed seasonal peaks of HFRS during Spring–Summer months from May to July, and

Autumn–Winter months (Fig. 1). The Autumn–Winter cycle was more extended, covering the period from late September to January. However, the majority of the cases occurred in the interval from October to December, amounting to 78% of all HFRS cases. This seasonal manifestation of HFRS morbidity is connected both with differing intensity of the contacts between humans and rodents, and with seasonal changes in rodent activity. In particular, much more intensive agricultural work takes place in April–May and September. In addition there is an intensive migration of *A. agrarius* rodents into dwellings with the coming of the cold season (September–October) along with an increase of hantavirus antigen-positive rodents during this period (Slonova et al., 1990b).

Although all age groups were involved, the HFRS epidemic cases among the population at the ages of 21–40 were most common (53% of the HFRS cases). Men accounted for 74% of the cases and women 26% of the cases.

Urban inhabitants contributed to 46% of the HFRS patients, the value being similar for both

years. Thirty-six percent of the HFRS patients were citizens of Khabarovsk; with those dwelling in ill-equipped houses in the suburbs and frequently visiting their country houses representing the highest incidence group. In addition to cases resulting from the seasonal migration of hantavirus-infected rodents (*A. agrarius*) into the houses, garbage dumps, ravines, and other habitats located within the city are favorable for year-round dwelling of *A. agrarius*.

3.2. Epidemiology of HFRS in 1996–1997 in the Primorsky region

During 1996 and 1997, 74 and 93 cases of HFRS, respectively, were recorded in the Primorsky region. Of these, 34 and 65 patients were inhabitants of Vladivostok, and 40 and 28 were from rural areas. Certain distinct features of hantaviral infection in Vladivostok compared to rural foci were evident. Men:women ratios were 1.4:1 in Vladivostok and 6:1 in rural areas. In 1996 and 1997, mild (29 and 31%) and moderate (65 and 63%) clinical cases were predominant in the urban patients of Vladivostok. In the rural area, severe cases of HFRS were recorded in 67 and 36% of the patients; moderate, in 33 and 59%; and mild, in 0 and 5% in 1996 and 1997, respectively. Seasonal dynamics of the HFRS morbidity in Vladivostok and the rural area of the Primorsky region differed from each other. Infection in Vladivostok was less typical with Winter–Spring peaks during

the 2 years (Fig. 2). The Winter–Spring increase, from February to May, comprised 63% of all the cases in 1996 and 57% in 1997. In contrast, in rural areas the peaks of morbidity from September to December (57% and 44%) were recorded in 1996 and 1997 (data not shown).

3.3. Phylogenetic analysis

One-hundred and ten blood samples were analyzed by RT-PCR using two sets of nested primers for the M segment, and 50 samples were analyzed by using two sets of nested primers for the S segment. From a total of 110 HFRS cases tested, 30 sequences of the G2-coding region of the M segment were identified (Table 2). S segment nested RT-PCR resulted in 15 hantavirus sequences, representing the N coding region. From nine samples, both M and S partial sequences were identified. Forty seven-percent of the samples from the Khabarovsk region and 16% from the Primorsky region were positive in RT-PCR analysis. A possible reason for the high percentage of negative results is that many of the blood samples had been thawed and refrozen several times, which would have reduced the quality of the viral RNA.

Analysis of the nucleotide and the corresponding amino acid sequences of the M segment region revealed three different groups of hantaviruses: two genotypes of HTN virus, FE and Amur (AMR), and one genotype of SEO virus, VDV.

Fig. 2. Seasonal dynamics of the HFRS morbidity in Vladivostok during 1996–1997.

Table 2
Genetic and serologic analysis of HFRS patient samples^a

Case	Location	Year	Clinical course	Day post onset ^b	Serum antibody titer (IFA) to				RT-PCR/segment ^c
					HTN	PUU	SEO	KBR	
3829	Kh, ss ^d	96	Moderate	6	1024	0	1024	0	FE/M
3844	Kh	96	Moderate	18	131 072	<16	16 384	512	FE/M
3850	Kh	96	Moderate	8	16 384	0	16 384	0	FE/M
3867	Kh	96	Moderate	8	32 768	NA	NA	NA	FE/M
3955	Kh	97	Severe	9	32 768	0	16 384	0	FE/M
4029	Vz	97	Severe	7	65 536	0	65 536	0	FE/S
4209	Kh, ns	97	Severe	11	32 768	64	16 384	64	FE/M
4211	Kh, ns	97	Moderate	4	4096	0	1024	0	FE/M,S
4226	P	97	Moderate	9	16 384	0	4096	0	FE/S
4228	Kh, ns	97	Moderate	5	2048	0	512	0	FE/M,S
4229	Kh, ss	97	Severe	4	128	64	64	0	FE/M
4231	Kh	97	Moderate	5	4096	0	1024	0	FE/S
4234	Am	97	NA	13	32 768	128	32 768	16	AMR/M
4235	P	97	Moderate	4	2048	0	512	0	FE/S
4241	Kh, ns	97	Mild	4	256	0	256	0	FE/M
4251	Kh, ss	97	Severe	10	13 1072	64	4096	256	FE/M
4253	Kh	97	Severe	9	8192	<16	4096	0	FE/M,S
4254	P	97	Moderate	8	4096	0	2048	0	FE/M,S
4290	Kh	97	Moderate	7	32 768	1024	32 768	1024	FE/M,S
4294	Kh, ss	97	Severe	13	16 384	4096	16 384	4096	FE/M,S
4296	Kh, ns	97	Severe	7	65 536	0	16 384	0	FE/M,S
4307	Lt	97	Severe	9	16 384	2048	16 384	512	FE/M
4309	Am	97	Severe	6	128	0	32	0	AMR/M
4313	Kp	97	Moderate	10	32 768	256	16 384	256	AMR/M
4320	Kh	97	Severe	10	16 384	0	16 384	512	FE/M,S
4329	Kh	97	Severe	7	32 768	1024	32 768	128	FE/M,S
4338	Lz	97	Severe	5	16 386	0	2048	0	FE/S
4382	Kh, es	98	NA	4	512	0	128	0	FE/M
7218	Vl	95	Moderate	6	128	0	128	NA	VDV/M
7303	Us	95	Moderate	17	2048	64	32	NA	FE/M
7406	Kr	95	Moderate	25	1024	32	NA	NA	FE/M
7649	Ks	96	Severe	21	2048	0	1024	NA	AMR/M
7701	Ch	96	Severe	7	1024	0	128	NA	FE/M
7732	Ar	96	Severe	11	512	0	64	NA	FE/S
7866	R	96	Moderate	16	512	0	64	NA	FE/M
8243	Vl	97	Moderate	10	512	0	2048	NA	VDV/M

^a Data shown are from all samples from which RT-PCR products could be recovered.

^b Day post onset for IFA analysis.

^c Positive PCR band/M or S segments/yielding FE, AMR, and VDV genotypes of virus nucleotide sequences; NA, no data available. Location name abbreviations are: Am (Amursk), Ar (Arseniev), Ch (Chernigovsk), Kh (Khabarovsk), Kp (Korphovsky), Kr (Kirovsky), Ks (Krasnoarmeisk), Lt (Litovko), Lz (Lazo), P (Pereyaslavka), R (Razdolnoye), Us (Ussuriisk), Vz (Vyazemsky), Vl (Vladivostok).

^d Suburbs of Khabarovsk: es, east; ns, north; ss, south.

The deduced amino acid sequences of 12 of the 24 FE RT-PCR products are identical to those of Hantaan virus, strains 76-118, HoJo and Lee, and the others had one to three possible substitutions (Fig. 3).

The amino acid sequences of the AMR genotype exhibited five amino acid differences relative to the HTN sequence. The VDV genotype had one amino acid difference in comparison with those of SEO virus.

The nucleotide and amino acid sequence comparisons of the partial M segment among three genotypes of Far Eastern hantaviruses are shown in Table 3. The nucleotide identity among the FE genotypes ranged from 94 to 100%; among the AMR genotypes, identities were 90.1–99.6%. Two VDV sequences were identical. The FE genotype

displayed a minimum of 15.9% nucleotide sequence dissimilarity with AMR genotype and 21.5% with VDV genotype. Comparing the partial M segment sequences of the new genotypes to those of the other hantaviruses revealed that the FE and AMR genotypes are hantaviruses most closely related to HTN virus, and the VDV geno-

Fig. 3. Amino acid comparison of partial G2 amino acids (248-333) among various hantaviruses.

Table 3
M segment nucleotide (bases 2735–2968) and amino acid sequence identities among three groups of Far Eastern hantaviruses and between Far Eastern hantaviruses and those from other countries^a

		Nucleotide (nt) and amino acid (aa) sequence identities (%)				
		Far Eastern FE	Far Eastern AMR	Far Eastern VDV	HTN	SEO
Far Eastern FE	nt	94.0–100	78.8–84.1	76.5–78.5	88.0–90.2	76.1–79.0
	aa	96.2–100	89.7–94.9	80.8–83.3	97.4–100	79.5–83.3
Far Eastern AMR	nt		90.1–99.6	75.2–79.8	82.5–84.2	75.6–79.4
	aa		98.7–100	82.1–83.3	93.6–94.9	82.1–83.3
Far Eastern VDV	nt			100	76.1	94.9–97.4
	aa			100	82.1	98.7

^a FE genotype includes samples: 3829, 3844, 3850, 3867, 3955, 4209,4211, 4228, 4229, 4241, 4251, 4253, 4254, 4290, 4294, 4296, 4307, 4320, 4329, 4385, 7303, 7406, 7701, 7866, AMR genotype includes samples: 4234, 4309, 4313, 7649, VDV genotype includes samples: 7218, 8243.

Table 4

S segment sequence identities (bases 605–923) among FE genotype of Far Eastern hantaviruses and between Far Eastern hantaviruses and those from other countries^a

		Nucleotide (nt) and amino acid (aa) homology (%)					
		FE	HTN	SEO	DOB	PUU	KBR
FE	Nt	94.3–100	87.4–89.3	62.1–63.4	61.8–63.7	47.3–51.7	49.8–52.1
	Aa	99.0–100	98.1–99.0	68.3–69.2	66.7–67.6	45.8–50.0	47.9–49.0

^a FE genotype includes samples: 4029, 4211, 4226, 4228, 4231, 4235, 4253, 4254, 4290, 4294, 4296, 4320, 4329, 4338.

type is most closely related to SEO virus (Table 3).

Sequence analysis of the partial S segments demonstrated over 94.3% nucleotide and 99% amino acid identities with each other and revealed a minimum of 10.7% nucleotide and 1% amino acid dissimilarity with HTN virus (Table 4). None of the samples of the AMR and VDV genotypes of the viruses was positive in RT-PCR for S segment.

Phylogenetic analysis of the partial M segment demonstrated three major lineages among hantavirus sequences (Fig. 4A). The first genotype of Far Eastern hantaviruses FE forms a new separate well-supported (98% bootstrap support) lineage within HTN virus clade, previously isolated from *A. agrarius* and humans in Korea. The four sequences from AMR genotype of the hantaviruses occupy a distinct highly supported lineage within the HTN virus clade, but phylogenetic relationships of these two lineages with other HTN lineages could not be fully resolved by this analysis (a weak 60% bootstrap support for one of the intermediate branches within the HTN clade). Phylogenetic analysis of aligned amino acid sequences fully resolved AMR lineage from other viruses within HTN clade (data not shown). Within the well-supported SEO virus clade, the VDV virus from Vladivostok occupies a new lineage of hantaviruses.

Unlike the M segment, analysis of the S segment more clearly reveals the phylogeny for the HTN clade (Fig. 4B). Phylogenetic relationships of the Far Eastern lineages were fully resolved from the prototype HTN 76-118. Two phylogenetically different lineages are found inside the FE lineage of hantaviruses. The 4290 isolate, included

earlier (Fig. 4A) in the FE lineage, groups together with the 4029 isolate and they both form a new, separate lineage (97% bootstrap support) within the HTN clade. Two lineages exhibited 4.4–6.9% differences in the nucleotide sequence from one another and at least 10.7% difference from the corresponding S genome fragment of HTN 76-118 (which is the hantavirus most closely related to the FE genotype; Table 4). On the S-tree there are no AMR and VDV sequences at all.

3.4. Serological results

To examine which hantavirus serotypes were involved in HFRS morbidity in the Primorsky region in 1996 and 1997, sera of the patients from Vladivostok ($n = 33$) and rural ($n = 21$) foci were titrated against HTN, SEO, and PUU viruses by PRNT (Table 5). Only those samples were presented in Table 5 from which RT-PCR product could be recovered. The data revealed 30 SEO and 24 HTN virus infections. No evidence for PUU virus infection was found. The neutralization results suggest that in 1996 and 1997, respectively, SEO virus caused 73% (8 out of 11) and 86% (19 out of 22) of the HFRS cases from Vladivostok and 17 and 11% of the rural cases.

Sera of 28 HFRS patients from the Khabarovsk region and of eight patients from the Primorsky region, from which RT-PCR products could be recovered, were compared for their reactivities with four hantavirus antigens (HTN, SEO, PUU, and KBR viruses) by IFA (Table 2). All of the samples tested were most reactive with the HTN and/or SEO virus antigens. Generally, the antibody titers obtained with the SEO virus anti-

Fig. 4. Phylogenetic relationship of Far Eastern hantaviruses to other characterized hantaviruses based on nucleotide sequence differences of (A) a 256-nt fragment of M segment (bases 2725-2981), and (B) a 356-nt fragment of S segment (bases 592-947).

B

Fig. 4. (Continued)

Table 5
Neutralization data of PCR positive human samples from Primorsky region^a

Case	Day post onset	PRNT antibody titer			RT-PCR
		HTN	PUU	SEO	
7218	15	32	0	1024	VDV
7303	21	256	0	0	FE
7406	26	1024	0	32	FE
7649	21	256	0	0	AMR
7866	28	256	0	0	FE
8243	21	128	0	256	VDV

^a Data shown are from samples from which RT-PCR products could be recovered and PRNT data are available.

Fig. 5. Geographic location of genetically identified HFRS cases. Location name abbreviations identified in Table 2. Symbol shapes correspond to virus genetic lineages identified in Fig. 4A. Distances are indicated by scale bar.

gen were either lower than the antibody titers to the HTN virus antigen or equal to them. One serum (8243) displayed fourfold higher reactivity to SEO virus antigen than to HTN virus antigen. Some of the sera (38%) also cross-reacted with the antigens of PUU and KBR viruses, the antibody titers to PUU and KBR virus antigens were considerably lower than to HTN and SEO virus antigens. Only one serum sample (4229) reacted equally well with SEO and PUU virus antigens.

4. Discussion

HFRS is endemic in the Far East of Russia, where 100–200 cases are reported annually. HFRS usually appears in severe and moderate forms with symptoms including abrupt onset, high fever, back pain, renal failure and hemorrhagic manifestations. This study presents evidence that at least two genetic lineages of HTN virus and one of SEO virus are the etiological agents of HFRS in the Far East of Russia.

The FE lineage was the most abundant in the samples that we tested, and was found throughout the territory. Amino acid sequences of the FE type differed only slightly in either the G2 or N regions examined as compared to prototype HTN 76-118. In addition, less than 6% variability in the nucleotide sequences of the partial M segment was observed within FE genotype. In some cases, genetic differences correlated with the geographic origin of samples. For instance, RT-PCR products from five clinical samples 4241, 4209, 4228, 4211, and 4296, which were collected at the same location or not farther than 15 km apart (Beryezovka village, north suburb of Khabarovsk), exhibited sequences, that were 99.6–100% identical. However, sequences obtained from samples from the south suburb of Khabarovsk (3829, 4229, 4251, 4294), collected not farther than 30 km apart from the north group, differed by 2.6–3.4%.

The AMR genotype, identified in four samples, forms a separate new lineage in the phylogenetic tree that is quite distinct from HTN viruses found in China and Korea. The partial M segment sequences of the AMR genotype showed at least 15.8% nucleotide sequence divergence compared to the respective segment of other hantaviruses (Table 3). Amino acid sequences of the AMR genotype of hantaviruses differed from the prototype virus by five amino acids in the region of the G2 protein studied. These differences were identical in all the samples, although the nucleotide sequences varied by up to 9.9%. Two of the amino acid differences (Ile to Val at positions 255, and 325) are identical to amino acids in SEO, DOB, KBR and PUU viruses.

This new AMR hantavirus genotype was identified in four disease cases: two patients in the Amursk district of the Khabarovsk region, one about 200 km from the first two cases, and the other about 400 km away, in the Primorsky region (Fig. 5). This previously unknown hantavirus genotype was named 'Amur' (AMR) according to its geographic origin, the area around the Amur River. There are no data at this stage to explain the lower frequency of this hantavirus in patients. The lower number of AMR-cases could be due to different rodent carrier, or lower prevalence, or different focus location of AMR virus. The latter

situation is less probable, as the cases recorded are rather geographically remote and one case is from a region of extensive sampling near Khabarovsk. The identification of the new AMR genotype viruses raises important questions concerning the rodent reservoir and distribution of the virus.

The presence of two genetically distinct variants of HTN virus in the Far East was not unexpected. Up to 16 distinct mammal hosts are associated with hantaviruses in the Far East of Russia. Four rodent species were identified as predominant members of the rodent assemblage and main carriers of hantaviruses: the striped field mouse *A. agrarius*, the Korean field mouse *A. peninsulae*, the gray-sided vole *Clethrionomys rufocanus*, and the reed vole *Microtus fortis* (Kosoy et al., 1997). *A. agrarius* is the most likely primary reservoir for FE genotype of viruses. The rodent host for the AMR genotype remains to be determined.

Cases of SEO virus infection were found in Vladivostok. The nucleotide sequences of the partial M segment identified in samples from two patients, infected in 1995 and 1997, were identical (and distinct from previously published SEO virus sequences). This is in accordance with the high genetic stability of SEO virus. One amino acid substitution was revealed in the partial G2 protein sequence of the VDV lineage as compared to other known SEO virus sequences. According to our neutralization data, SEO virus causes about three-fourths of the HFRS cases in Vladivostok, and 14.3% of rural cases, suggesting an important epidemiological significance of SEO virus in the Primorsky region. These data correlate with those from a previous study of community structure and prevalence of hantaviral infection in rodents in this region. In that study, *R. norvegicus* were identified in the southern part of the Primorsky region and the percent of hantavirus antigen-positive animals varied from 9.1 to 12.5 (Kosoy et al., 1997). The VDV cases were recorded in patients with moderate HFRS.

We analyzed the correlation between the severity of the HFRS caused by FE genotype of HTN virus and the amino acid structure of the genomic fragment studied. Two subgroups were apparent in the FE lineage: one group causing predominantly moderate and another causing severe HFRS. The

first group included hantaviruses with identical sequences in the G2 region to those of the prototype strain 76-118 (13 cases). They were detected in the following groups of patients: 23% from severe cases; 69% from moderate; and 8%, from mild. Hantaviruses of this group have been isolated predominantly from the patients with moderate form of HFRS. The hantaviruses having the Met to Thr substitution at position 262 of G2 were found in eight patients; six of whom had the severe form of HFRS (Table 2, Fig. 3). In addition, these patients had antibodies that reacted with HTN/SEO/PUU/KBR virus antigens. These findings are in line with the idea that changes in the viral genome produce changes in pathogenicity. Whether these specific changes are relevant to disease severity awaits further studies.

In conclusion, our study indicates that at least three distinct lineages of hantaviruses appear to be maintained in the Far East of Russia: FE and AMR genotypes of HTN virus and VDV genotype of SEO virus. Further studies will be required to determine if these lineages are variants of HTN and SEO viruses or if they are sufficiently distant to constitute definition as distinct hantaviruses.

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