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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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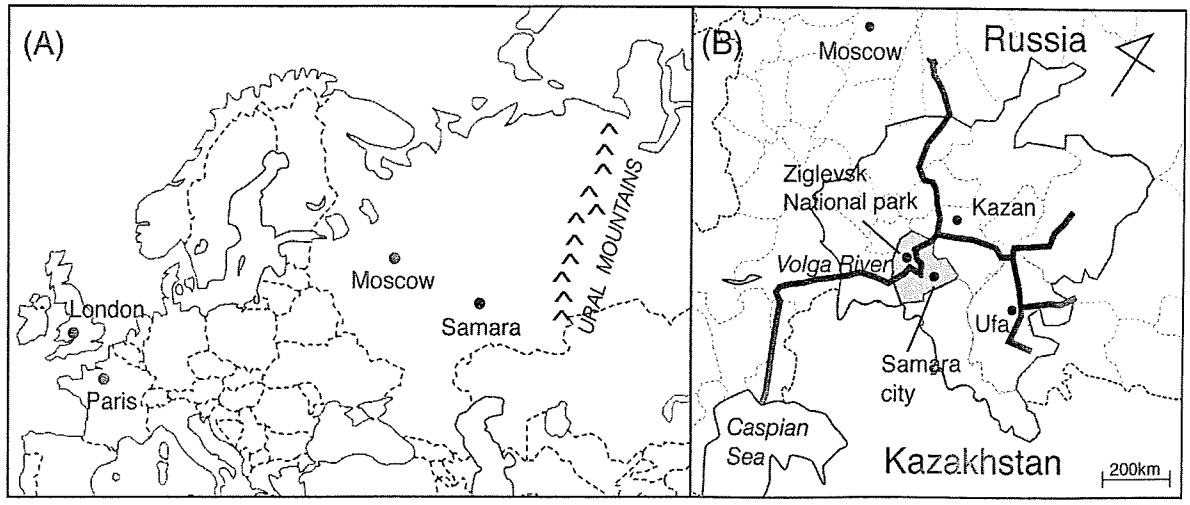

Fig. 1. Map of the survey site. (A) European Russia and European countries. (B) Samara region in the Volga River area; Samara region.

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 *n*g 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 PUUV	IFA titer HTNV	IFA titer SEOV	Virus RNA[a] 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-

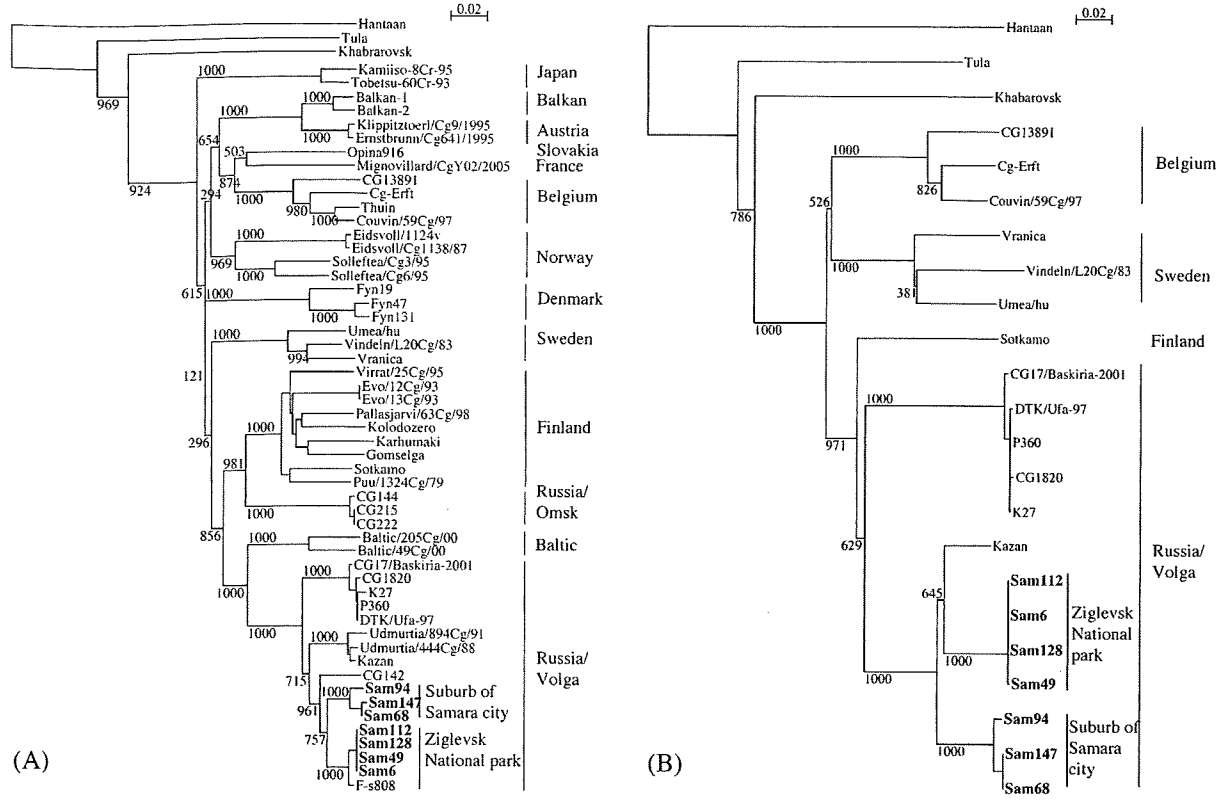

Fig. 2. Hantavirus phylogenetic trees. Hantavirus sequences were aligned using Clustal X, and the phylogenetic trees were generated using the neighbor-joining method. The reliability of the dendrogram was evaluated by 1,000 bootstrap replicates. (A) Phylogenetic tree of hantavirus partial S-segments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

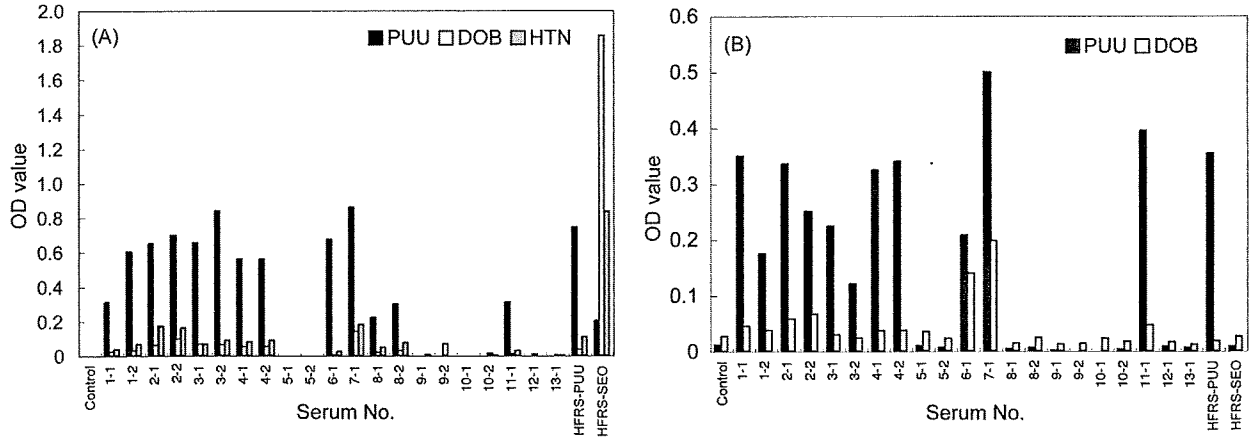

Fig. 3. Detection of anti-hantavirus antibodies in HFRS patients and patients with high fevers of unknown etiologies by ELISA. Baculovirus-expressed hantavirus nucleocapsid proteins (NPs) were used as the antigens. PUU: full length NP of PUUV; DOB: full length NP of DOBV; HTN: full length NP of HTNV; 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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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.

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.

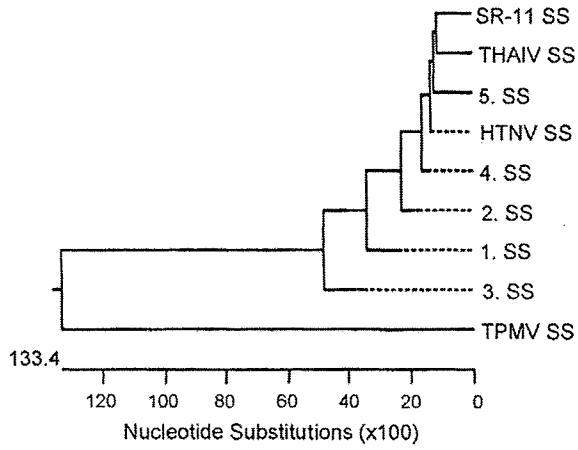

Figure 1 Phylogenetic tree construct of the partial S genome segment from patient samples. SS: S segment; SR-11: Seoul virus; THAIV: Thailand virus; HTNV: Hantaan virus; TPMV: Thottapalayam virus; 1, 2, 3, 4 and 5 represent the partial S segment from patient samples.

In this study, the mean number of days post onset of illness at which hantavirus RNA was detectable was 9 days (Table 1). One patient (#19) who was positive for hantavirus RNA had very low levels of second round product and the products could not be sequenced. In the remaining five patient samples that were positive for hantavirus RNA, the PCR products after a nested RT-PCR were sequenced and a phylogenetic tree was constructed. The nucleotide sequences were compared with S genome sequences of HTNV, SEOV, THAIV and TPMV. The phylogenetic analyses of the sequences are shown in Figure 1. The nucleotide identity of human sequences with that of HTNV ranged from 84% to 98.5%, with SEOV from 69% to 78.5%, with THAIV from 61% to 71.7% and with TPMV from 8.2% to 30%. The highest homology of the human sequences was with HTNV. Among the sequences from patient samples there was a nucleotide homology of 84–98.5%. Figure 2 shows the seasonal distribution of hantavirus cases in our study. Most of the cases were distributed in the early (spring) cool months and later (autumn) rainy months of the year.

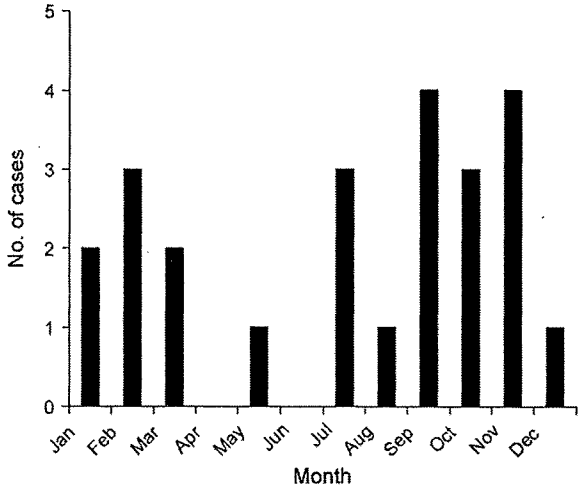

Figure 2 Cumulative seasonal distribution of hantavirus cases during 2005–2007, showing clustering of hantavirus cases in the early and latter part of the year.

4. Discussion

HFRS remains undefined in South Asia largely due to the unavailability of sensitive and specific serodiagnostic assays. IFAs and ELISA systems employing recombinant antigens are widely used. Hantavirus RNAemia is short lived and in some cases undetectable, thus the use of RT-PCR for hantavirus diagnosis is limited. Whatever the diagnostic tools used, reliability of results is an issue in areas where the circulating serotypes are largely unknown, hantavirus cases have not been documented and the extent of hantavirus-related disease is unknown. The present study was performed to expand our knowledge of hantavirus-related acute febrile illness in India and to advocate judicious use of serological and molecular techniques to improve hantavirus diagnosis.

Serological assays for hantavirus diagnosis in India should include antigens of HTNV, DOBV, PUUV and TPMV as several distinct hantaviruses cause HFRS in the Old World. Serology based on IgM ELISA is effective in the rapid diagnosis of hantavirus infections in endemic regions of the world.

In this study, 78 patients (22.5%) tested positive for anti-hantavirus IgM by ELISA and IFA, only 18 of whom had detectable levels of hantavirus-specific IgG. Although the demonstration of both IgM and IgG hantavirus-specific antibodies enables reliable serological confirmation of hantavirus infection, immune responses could differ with the infecting hantavirus serotype. Delayed IgM response in nephropathia epidemica (NE) has been reported and in approximately 5–10% of NE patients detectable levels of IgG were not seen at the time of admission.[19] In the absence of a μ-capture ELISA, stringent serological criteria such as those adopted in this study have the limitation of missing cases with a delayed IgM/IgG response. This would also explain the absence of anti-hantavirus IgM in two patients who were positive for hantavirus RNA.

Differences in the type of antigens used in the assays (ELISA and IFA) and/or the circulation of unknown hantavirus serotypes in the region could explain the differences in reactivities seen in the two assays.

These molecular findings correlate well with results of a serotyping study which indicated that the circulating serotypes are predominantly HTNV-like as well as the presence of Thailand and a non-typeable Hantaan-like species in India. The reactivity pattern of serotyping appears to suggest the presence of more than a single novel hantavirus species in India (our unpublished data). However, these results are preliminary and a complete picture must await characterisation of circulating serotypes with primers designed to detect specific circulating serotypes/genotypes.

Sequences obtained from patients were very divergent from TPMV, the only documented serotype from India. At this juncture we would not be able to comment on these results as the divergence of TPMV from other hantaviruses warrants the use of novel serological and molecular assays for its detection.

There were a few limitations of this study. Owing to the unavailability of antigens, it was not possible to com-

pare the reactivity of samples with individual antigens of different serotypes, which would have given a better picture of the circulating serotype. The size of the second round product was small and novel primers for larger amplicons await design. We could not obtain convalescent sera from most of the patients. TPMV is an important isolate from India and needs to be included in serological panels, as cross-reactivity between TPMV and other hantaviruses is reportedly low.

Approximately 20% of acute HFRS cases are positive for hantavirus RNA[20] and thus molecular diagnosis should be used only to complement serodiagnostic assays. RT-PCR may show false negativity as the viraemia in hantavirus infections is short. Most of the patients with febrile illness attending our clinics seek medical care around a week after the onset of illness. Samples are best suited for demonstration of hantavirus RNA when collected within 5–7 days after the onset of illness.

All infectious agents carried by rodents or ectoparasites of rodents should be considered in tandem. Co-infection with *Leptospira* and hantavirus has been reported.[21] In our study, three patients showed co-infection with hantavirus and leptospirosis whilst two were co-infected with dengue and hantavirus.

The temporal distribution of cases can also be correlated with the distribution of seasonal fluctuations in rodent populations in India. In South Asia, some of the bandicoot (giant rat) species (genus *Bandicota*) have two peaks in breeding activity (April and August–September[22]) and population densities of rats as documented are greatest from September–November and lowest from May–July.[23]

This study complements our previous reports on hantavirus activity in India. It establishes the likelihood of hantavirus infections among patients with acute febrile illness. Since the clinical features of hantavirus infections are not pathognomonic and laboratory work-up is essential for confirmation of the diagnosis, it is important to establish cost-effective, user-friendly diagnostic tools (serological and molecular) for rapid diagnosis of hantavirus infections in humans.

Authors' contributions: GS and PA designed the study protocol; SC carried out the immunoassays and drafted the manuscript; KY and JA supplied IFA slides and helped analyse the results obtained; HKB, AC, KT and AP carried out the clinical assessment, with the identification of subjects and collection of samples. All authors read and approved the final manuscript. GS is guarantor of the paper.

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Conflicts of interest: None declared.

Ethical approval: Institutional Research Ethics Committee of the Christian Medical College, Vellore, Tamil Nadu, India (R.C. Min. No. 5838 dated 21 February 2006) and the Indian Council for Medical Research (ICMR).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.trstmh.2009.01.016.

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Brief Communication

HANTAVIRUS SPECIES IN INDIA: A RETROSPECTIVE STUDY

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Abstract

Hantaviruses cause hemorrhagic fever with renal syndrome in Europe and Asia. There are about 20 documented hantavirus species and newer species are being described worldwide, especially in non-rodent reservoirs, i.e shrews. Focus reduction neutralization test is the classical serotyping technique for hantavirus. However, this study employs a previously established serotyping ELISA, to retrospectively analyze known hantavirus IgG reactive samples for infecting serotypes. The result suggests presence of *Thailand virus*-like and *Hantaan virus*-like strains in India.

Key words: *Hantavirus, India, serotypes*

Introduction

Hantaviruses are rodent-borne viruses belonging to the *Bunyaviridae* family. They cause hemorrhagic fever with renal syndrome (HFRS) in Asia and Europe and hantavirus cardiopulmonary syndrome (HCPS) in the Americas. Hantaan virus (HTNV), Seoul virus (SEOV), Dobrava virus (DOBV) and Puumala virus (PUUV) are hantavirus serotypes circulating in Europe and Asia. HTNV and DOBV-related HFRS cases are severe while SEOV, which is hosted by rats, causes mild HFRS with a low mortality rate of 1-2%. Nephropathia epidemica (NE), a mild form of HFRS, is caused by PUUV in Scandinavia.[1] There have been some recent reports of Thailand virus (THAIV)-related HFRS.[2]

The only hantavirus serotype indigenous to India is the *Thottapalayam virus* (TPMV) which was isolated from a shrew, *Suncus murinus*, in 1964.[3] The lack of reactivity of the nucleocapsid (N) protein-specific monoclonal antibodies raised against HTNV and PUUV with recombinant TPMV N proteins produced in *E. coli* or yeast[4] (Mertens *et al.*, unpublished data) proves the phylogenetic and antigenic diversity of TPMV.

Serological evidence of hantavirus infections have been documented from India[5-7] but the circulating species are unknown. We report preliminary data on the serotyping of hantavirus species in India by testing hantavirus reactive human sera using truncated nucleocapsid N protein derivatives of different Asian hantaviruses.

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Materials and Methods

Twelve samples were subjected to serotyping ELISA. Samples number 1, 3 and 12 were negative and the other nine (sample numbers 2, 4 to 11) were positive for anti hantavirus IgG antibodies. Criteria for anti hantavirus IgG reactivity were as per our previous publication. [6] All serotyped samples (n=12) were initially tested by two assays; a commercial anti hantavirus IgG ELISA (Focus Technologies Cypress, California, USA) and indirect immunofluorescence assay (IFA) using HTNV-infected Vero E6 cells. All nine seropositives were confirmed by Western blot (WB) analysis using a recombinant protein of HTNV (Fojnica strain). Except sample number 7, all others were part of a seroprevalence study on hantavirus infections in India and originated from patients with chronic renal disease (sample numbers 1 to 6) and healthy blood donors (sample numbers 8 to 12); (Fig. 1).[6] Sample number 7 was from a patient with suspected hantavirus-like disease. Samples were chosen for serotyping based on their availability and level of reactivity in the ELISA and IFA as shown in Table 1. Cumulative data on reactivity of these samples have been presented in our seroprevalence report.[6]

Results

Results of 12 serotyped samples are represented in Fig. 1.

All samples were characterized using the entire recombinant N proteins of HTNV (HTNV antigen complex) and PUUV (PUUV antigen complex; Fig. 1). In addition, to differentiate between hantavirus serotypes, truncated and recombinant N proteins (trNPs) of HTNV, SEOV and THAIV lacking 49 amino acids in the amino-terminal region of the N protein and expressed by a baculovirus system were used as ELISA antigen for serotyping.[8,9] The cut-off OD of the serotyping ELISA was calculated using negative

Table 1: Summary of serological results (anti-hantavirus IgG reactivity) of samples used for hantavirus serotyping

Sample ID	ELISA Index	IFA	WB	Serotyping
#1	1.6	N	N	N
#2	1.2	3+	P	P
#3	2.7	N	N	N
#4	3.5	3+	P	P
#5	1.12	2+	P	P
#6	2.0	>3+	P	P
#7	2.9	>3+	P	P
#8	2.2	4+	P	P
#9	2.6	>2+	P	N
#10	1.5	3+	P	N
#11	1.4	>2+	P	P
#12	1.3	N	N	*

P- Positive, N-Negative. A sample was considered positive for anti-hantavirus IgG if reactive by both ELISA and IFA. An ELISA Index > 1.10 and an IFA reactivity ≥ 2+ were considered positive. *This sample though non-reactive with HTNV complex antigen showed reactivity with SEOV serotyping antigen. Samples #1 to #6 Patients with chronic renal disease. Samples #8 to #12 Healthy blood donors

sera and was less than 0.1. However, to eliminate false positive reactions, 0.2 was taken as cut-off OD. Two human sera that were negative for hantavirus-specific antibodies are represented in Fig. 1 as NHS-1 and NHS-2. Positive control sera from patients infected with HTNV, SEOV, THAIV and PUUV have been used as positive controls and demonstrated strongest reactivity with the corresponding homologous antigen (Fig. 1).

Seven of the nine screening positive sera, numbers 2, 4, 5, 6, 7, 8 and 11 were found reactive with the entire HTNV antigen complex. Sera numbers 9 and 10, although detected in the screening assays, were non-reactive with this antigen. None of the nine sera showed a significant reactivity with the entire PUUV N antigen complex. The causative hantavirus species that the seven individuals were exposed to belongs to the HTNV complex. Sera numbers 2 and 6 showed reactivity with trNP of THAIV antigen suggesting infection with a THAIV-like virus. Sera numbers 4 and 5 could not be serotyped using trNP of HTNV, SEOV and THAIV suggesting past infection with an unknown HTNV-like virus.

Sample number 7, from a patient with suspected hantavirus-induced disease, although strongly reactive with the entire HTNV antigen, showed no reactivity with any of the serotyping antigens suggesting an infection with a virus of the HTNV complex. This serum originated from a 13-year-old patient who presented with fever, headache, myalgia and cough at a peripheral hospital. However, virus-specific IgM was not detected (data not shown). Therefore,

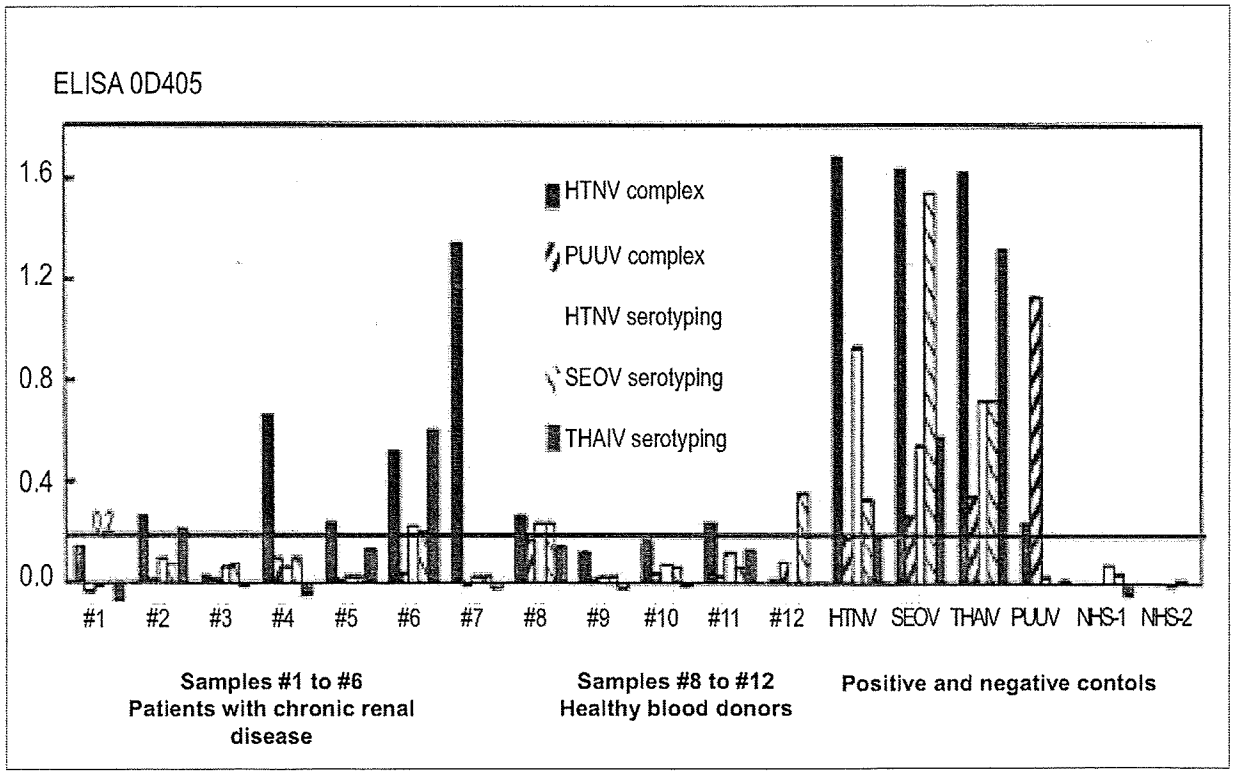

Figure 1: Profiling of anti-hantavirus antibody in a serotyping ELISA Sample #7 was from a patient with hantavirus-like illness. Except samples #1, #3 and#12, all samples were anti-hantavirus IgG reactive in ELISA, IFA and WB. The positive control sera originated from HTNV- infected, SEOV- infected, THAIV-infected and PUUV-infected patients. The negative control sera NHS-1 and NHS-2 were non reactive to the antigens used

the individual may have been infected with hantavirus previously. Unfortunately, no information is available about the patient's living conditions or his exposure to rodents.

The blood donor samples reactive by the serotyping ELISA (numbers 8 and 11) showed only low levels of anti-hantavirus IgG antibodies. This low level of anti-hantavirus antibodies might be due to an infection long time ago, or perhaps by a distantly-related hantavirus. Sample number 8 showed similar OD values with entire HTNV N protein and trNP of HTNV and SEOV. The reactivity pattern of serum number 11 may indicate, similar to sera numbers 4 and 5, a previous infection with an unknown HTNV-like virus.

In general, the three negative sera; numbers 1, 3 and 12 did not react significantly with any of the N antigens used. The only exception was serum number 12 reacting with the trNP of SEOV. Similarly numbers 9 and 10, though detected in the screening assays, were non- reactive with the entire HTNV antigen complex. These results may indicate limitations in the sensitivity of the screening assays used and of the entire N protein of HTNV-based ELISA, also; alternatively a specificity problem of the SEOV serotyping ELISA.

Discussion

Through this serological study it appears that at least two hantavirus species seem to circulate in India: the THAIV–like virus and perhaps one or more unknown HTNV-like viruses. However, we cannot exclude that the infections in subjects numbers 4 and 7 and perhaps numbers 5 and 11 are caused by a HTNV strain that contains amino acid exchanges in the N protein causing the unexpected lack of reactivity with trNP of HTNV. The reactivity pattern of the serum samples with the different serotyping antigens seems to clearly suggest the presence of more than a single novel hantavirus species in India.

Sample number 6 showed good reactivity with THIAV serotyping antigens and with HTNV complex antigen but low reactivity with HTNV and SEOV serotyping antigens. The difference in the OD is suggestive of infection with THAIV or THAIV-like serotype. Sample number 8 shows low reactivity with HTNV complex antigen, HTNV and SEOV serotyping antigens. This kind of reactivity could suggest presence of a non typeable HTNV-like serotype.

THAIV is pathogenic to humans and documented to cause HFRS in Thailand.[2] The known reservoir for the THAIV serotype, *Bandicota indica*, is also distributed in India. The reactivity pattern of numbers 2 and 6 may represent human THAIV infections in India.

The findings presented in this paper are preliminary. The differentiation of the etiologic species is relevant in epidemiological terms as the disease severity of hantavirus infections depends on the hantavirus species/serotypes. Further studies including larger human serum panels as well as studies in rodent and shrew reservoirs are needed for more conclusive results on the circulating hantavirus species India. Future serotyping studies should include testing dilutions of sera. In future it is also important to conduct serological surveys using immunoassays that incorporate antigens of TPMV.

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

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Coxiella burnetii Isolates Cause Genogroup-Specific Virulence in Mouse and Guinea Pig Models of Acute Q Fever[▽]†

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Q fever is a zoonotic disease of worldwide significance caused by the obligate intracellular bacterium *Coxiella burnetii*. Humans with Q fever may experience an acute flu-like illness and pneumonia and/or chronic hepatitis or endocarditis. Various markers demonstrate significant phylogenetic separation between and clustering among isolates from acute and chronic human disease. The clinical and pathological responses to infection with phase I *C. burnetii* isolates from the following four genomic groups were evaluated in immunocompetent and immunocompromised mice and in guinea pig infection models: group I (Nine Mile, African, and Ohio), group IV (Priscilla and P), group V (G and S), and group VI (Dugway). Isolates from all of the groups produced disease in the SCID mouse model, and genogroup-consistent trends were noted in cytokine production in response to infection in the immunocompetent-mouse model. Guinea pigs developed severe acute disease when aerosol challenged with group I isolates, mild to moderate acute disease in response to group V isolates, and no acute disease when infected with group IV and VI isolates. *C. burnetii* isolates have a range of disease potentials; isolates within the same genomic group cause similar pathological responses, and there is a clear distinction in strain virulence between these genomic groups.

Coxiella burnetii, the etiologic agent of acute and chronic Q fever, is an obligate intracellular bacterium with worldwide distribution and a diverse host range. Livestock serve as the organism's primary reservoir and may be asymptomatic carriers or exhibit reproductive disorders. Ticks are important in the maintenance of the disease in nature and have been shown to transmit the infection transovarially (37). Humans are most often infected through inhalation of the bacterium in fine-particle aerosols, though transmission may also occur through ingestion of the organism from contaminated, unpasteurized dairy products (22, 27). Although a high percentage of infections may result in subclinical or asymptomatic infection, humans can become ill from exposure to as few as 10 organisms (6) and may display signs of (i) an acute flu-like illness with or without pneumonia and/or hepatitis (30, 31) or (ii) a chronic disease manifesting most frequently as endocarditis and/or hepatitis (40, 41).

C. burnetii isolates have been obtained from natural Q fever infections in humans and other animals. Several theories have been proposed to explain the dichotomy in development of acute and chronic Q fever. Unique sequence differences between genomic groups are correlated with the clinical expression of Q fever (44). Biochemical markers have grouped *C. burnetii* isolates from chronic-disease patients separately from acute-disease/arthropod/domestic animal isolates, but whether these groupings predict virulence potential and acute/chronic-disease outcomes has not yet been fully resolved (20). Samuel et al. were the first to separate these isolates and their resulting diseases based on plasmid patterns (44). Hackstadt used variations in lipopolysaccharide (LPS) banding patterns to divide isolates of *C. burnetii* into three groups, and group distinction was noted in correlation with acute or chronic disease (16). Hendrix et al. separated *C. burnetii* isolates into six genomic groups (20). Group I to III isolates have a QpH1 plasmid and have been isolated from ticks, acute human Q fever cases, cow's milk, and livestock abortions. Groups IV and V have a QpRS plasmid or no plasmid (with plasmid-related sequences integrated into the chromosome), respectively, and have been associated with livestock abortions and human chronic endocarditis or hepatitis. Group VI isolates were collected from wild rodents in Dugway, UT, and were infectious but avirulent in rodent models of disease (47, 48). Jager et al. used restriction fragment length polymorphism (RFLP) to differentiate 80 *C. burnetii* isolates and reproduced distinguishable patterns for reference isolates in groups I, IV, V, and VI (23). More recently, multiple-locus variable nucleotide tandem repeat analyses (49) have validated these groupings. Infrequent-restriction-site PCR of 14 livestock and tick isolates resulted in six groups; subsequent multiple-locus variable-number tandem repeat analysis typing of 42 isolates revealed 36 genotypes (2). Glazunova et al. used multispacer sequence typing to analyze 173 isolates, a majority of which were acquired from chronic-

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