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Short communication

Puumala hantavirus in Slovenia: Analyses of S and M segment sequences recovered from patients and rodents

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Abstract

In Slovenia, the co-existence of Dobrava and Puumala (PUUV) hantaviruses in a single endemic region has been demonstrated. This study presents selected Slovenian HFRS cases caused by PUUV combined with genetic analysis of viral genome sequences recovered from clinical specimens and tissue samples of *Clethrionomys glareolus* (bank voles). Serum samples from nine HFRS patients were included in the study. Rodents study sites were selected with regard to the HFRS cases. Partial S segment sequences were recovered from all nine patients and partial M segment sequences could be recovered from seven. Partial S and M segments sequences were also recovered from five *C. glareouls* captured at three different study sites. The sequences from Slovenian clinical specimens and rodent tissue samples belonged to the PUUV genotype and formed a distinct genetic lineage of PUUV. Human and rodent PUUV sequences located in the closest proximity to each other on the phylogenetic trees suggest genetic links between the human cases and the hantaviral strains circulating in natural foci of this zoonotic infection. Analysis of the complete S segment sequences recovered for two wild-type PUUV strains confirmed the existence of a distinct genetic lineage and also indicated a possible quasispecies type of Slovenian PUUV.

Keywords: Hantavirus; Puumala virus; Dobrava virus; Serological and genetic analyses; Slovenia

In Europe, three hantaviruses pathogenic for humans are well-documented. PUUV carried by *C. glareolus* (bank vole) and causing a milder form of HFRS is reported throughout Europe and western Russia (Brummer-Korvenkontio et al., 1982). DOBV is carried by *Apodemus flavicollis*, the yellow-necked filed mouse, and is associated with a severe form of a disease with up to 12% mortality in the Balkans (Avsic-Zupanc et al., 1999; Papa et al., 1998). SAAV is carried by *Apodemus agrarius*, the striped field mouse, and is found in the Baltic and Central Europe causing mild HFRS similar to PUUV infection (Golovljova et al., 2002; Lundkvist et al., 2002).

In Slovenia, both severe and mild clinical courses of the disease have been observed, with an overall lethality rate of 4.5% (Avšič-Županc and Petrovec, 2003). We have demonstrated that DOBV and PUUV co-exist in a single endemic region of Slovenia and are capable of causing HFRS with significant differences in severity (Avsic-Zupanc et al., 1999). Earlier epidemiological surveys indicated that *A. flavicollis* and *C. glareolus*, which are common rodent species throughout central Europe, were most often infected with hantaviruses (Avsic-Zupanc et al., 1989, 1995, 1992). Furthermore, results of genetic analysis of wild-type (wt) DOBV from Slovenia indicated that DOBV carried by its natural host, *A. flavicollis*, has a significant geographical clustering of genetic variants. In contrast, the *A. agrarius*-harbored SAAV, found to co-circulate within the same geographical location as DOBV without mixing, exhibit notable genetic and phylogenetic differences (Avsic-Zupanc et al., 2000). The purpose of this study was to present selected HFRS cases caused by PUUV in Slovenia with direct genetic detection of PUUV genome sequences

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Table 1
Description of HFRS patient samples and ELISA, FRNT and RT-PCR results

Patient	Location[a]	HTNV ELISA		PUUV ELISA		DOBV ELISA	FRNT			PCR	
		IgG	IgM	IgG	IgM	IgG	HTNV	PUUV	DOBV	S[b]	M[c]
P1	NM	neg	400[d]	800	>3200	neg	<40	80[e]	<40	+[f]	+
P2	NM	neg	400	1600	>6400	neg	<40	320	<40	+	+
P3	NM	neg	800	800	>6400	neg	<40	80	<40	+	–[g]
P4	LJ	200	800	1600	12800	neg	<40	320	<40	+	–
P5	LJ	neg	800	1600	3200	neg	<40	640	<40	+	+
P6	LJ	neg	800	800	>6400	100	<40	320	<40	+	+
P7	MS	400	400	6400	3200	800	<40	5120	40	+	+
P8	MS	neg	400	400	>6400	neg	<40	80	<40	+	+
P9	MB	400	200	800	12800	200	40	320	<40	+	+

[a] Geographical region of selected HFRS patients (NM-Dolenjska region; LJ-surrounding of Ljubljana; MS-Prekmurje region).
[b] S segment.
[c] M segment.
[d] Reciprocal value of the ELISA titer.
[e] Reciprocal value of the FRNT titer.
[f] Positive PCR result.
[g] Negative PCR result.

recovered from patient specimens and from tissue samples of *C. glareolus*.

Acute serum samples from nine selected HFRS patients were included in the present study. During the course of the disease the clinical diagnosis was confirmed serologically by testing acute-phase serum samples by enzyme-linked immuno assay (ELISA) IgM and IgG tests as described previously (Avsic-Zupanc et al., 1999). To identify the serotype of the infecting hantavirus, end-point titers of neutralizing antibodies in convalescent sera (drawn ≥1 month after the onset of disease) were determined by focus reduction neutralization test (FRNT) as described earlier (Lundkvist et al., 1997).

Rodents were trapped in selected study sites with regard to reported HFRS cases. Sera collected from rodents were initially screened for the presence of IgG antibodies to hantaviruses by ELISA. All positive sera were confirmed by an indirect immunofluorescence assay (IFA) (Avsic-Zupanc et al., 2000).

RT-PCR was performed on samples from nine serologically confirmed PUUV patients and on all seropositive rodent samples by using primers MOF103 and MOR204, which amplify a 490-bp region from the M segment as described previously (Avsic-Zupanc et al., 2000). A second-round PCR (for nt 1296–1620) was carried out using PUUV-specific primers that reside within the amplified region of the RT-PCR primers in a "nested" fashion (Chu et al., 1995). Previously described nested primers (Bowen et al., 1997), designed to detect all known hantaviruses associated with rodents of the *Arvicolinae* subfamily, were used to obtain partial S segment sequences. RT-PCR for the entire S segment was performed on two selected rodent samples essentially as described before (Plyusnin et al., 1994a,b). PCR-amplicons corresponding to the entire S segment were cloned into pGEM-Teasy vector (Promega, Madison, WI).

All PCR products and recombinant plasmids with the complete cDNA copy of the viral S genome segment were sequenced automatically using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit or M13F and M13R Dye Primer sequencing kits with AmpliTaq DNA Polymerase FS (PE Applied Biosystems, Foster City, CA). The chromatograms were analyzed and assembled using the Staden package software (MRC Laboratory of Molecular Biology, Cambridge, UK) run on the Linux Operating System.

Multiple nucleotide alignments were prepared manually using the SeqApp 1.9a169 sequence editing program. Phylogenetic analysis was performed using the PHYLIP program package (Felsenstein, 1993) and TreePUZZLE (Strimmer and von Haeseler, 1996). In PHYLIP, 500 bootstrap replicates (SEQBOOT program) were fed to the distance matrice algorithm (DNADIST, with either Felsenstein84 or Kimura80 option); distance matrices were analyzed with the Fitch-Margoliash (FM, FITCH) or Neighbor-joining (NJ, NEIGHBOR) tree-fitting algorithm; the bootstrap support values were calculated with the CONSENSE program. In TreePUZZLE, the Hasegawa-Kishino-Yano-85 model was used with 10,000 puzzling steps; base frequencies were estimated from the datasets.

Patients were selected based upon the hantavirus causing the disease and the HFRS endemic region within the country. Three well-known HFRS endemic regions were included: Dolenjska (NM), Ljubljana and surroundings (LJ) and Prekmurje (MS). In all nine patients hantavirus infection was confirmed in acute-phase serum samples by the presence of virus-specific IgM antibodies using ELISA (Table 1). FRNT end-point titration of convalescent serum samples of all nine patients confirmed PUUV infection. The results were in complete agreement with the typing outcome as initially indicated by ELISA. Furthermore, seven acute serum samples were positive when a portion of the M segment region was amplified by nested RT-PCR. Amplicons of the expected size were obtained from all nine serum samples when nested RT-PCR of the S segment was performed.

A total of 367 rodents were trapped at four study sites during a 6 year period, representing five rodent species. For the purpose of these studies only bank voles—*Clethrionomys glareolus* (93 specimens) and filed voles—*Microtus agrestis* (14 specimens), were further analyzed. Seropositive *C. glareolus* were found in three out of four studied areas in a range from 21.2 to 50%.