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Short communication

Detection of Dobrava hantaviruses in *Apodemus agrarius* mice in the Transdanubian region of Hungary

Ferenc Jakab [a,b,*], Győző Horváth [a], Emőke Ferenczi [c], Judit Sebők [d], Zoltán Varecza [e], György Szűcs [b]

[a] *Institute of Biology, University of Pécs, Pécs, Hungary*
[b] *Baranya County Institute of State Public Health Service, Regional Laboratory of Virology, Pécs, Hungary*
[c] *National Center for Epidemiology, Department of Viral Diagnostics, Reference Laboratory of Viral Zoonoses, Budapest, Hungary*
[d] *2nd Department of Medicine and Nephrology, University Hospital, Pécs, Hungary*
[e] *Department of Immunology and Biotechnology, Faculty of Medicine, University of Pécs, Pécs, Hungary*

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Abstract

Dobrava hantavirus (DOBV) belongs to the genus *Hantavirus* of the family *Bunyaviridae*, and is carried by yellow necked and striped field mice (*Apodemus flavicollis* and *Apodemus agrarius*), respectively. The aim of this study was to detect and genetically characterize new DOBV strains in rodents captured in the Transdanubian region of Hungary. Rodent corpses were dissected and lung tissues were used for hantavirus detection by SYBR Green-based real-time RT-PCR using specific primers located in the S-segment of the virus genome. A total of 22 captured animals of the *Apodemus* species were tested for the presence of DOBV. Three out of the 22 mice were positive. Phylogenetic and molecular sequence analyses showed that Hungarian DOBVs were most closely related to those viruses detected from *A. agrarius* mice in Slovenia. Based on our new data from the region we concluded that extended reservoir studies would be necessary in the future.

Keywords: Dobrava–Belgrade hantavirus; Real-time RT-PCR; Hungary; Phylogenetic analysis

Dobrava hantavirus (DOBV) belongs to the genus *Hantavirus* of the family *Bunyaviridae*, and is carried by yellow necked (*Apodemus flavicollis*) and striped field (*Apodemus agrarius*) mice (Nemirov et al., 2002). The virus was first described from Slovenia and Yugoslavia (Avsic-Zupanc et al., 1992; Gligic et al., 1992). Thereafter, DOBVs were detected in many European countries, including Slovakia, Greece, Germany, Croatia and Serbia-Montenegro (Vapalahti et al., 2003). Infection is transmitted to humans by rodents. The virus may cause severe hemorrhagic fever with renal syndrome (HFRS) (Krüger et al., 2001). The most common symptoms of infection are fever, muscle and joint pain, and kidney dysfunction. Petechial rash, severe hemorrhage and pulmonary failure may also occur (Krüger et al., 2001; Schütt et al., 2004). The goal of this study was to detect and characterize the genetic lineages of DOBV occurring in the Transdanubian region of Hungary.

Apodemus rodents were screened to detect DOBVs. Small mammals were trapped at three rural locations (near Gyékényes, Görcsöny and Sármellék villages); (Fig. 1) where ecological studies and population monitoring were conducted. Rodents were trapped during the summer and autumn of 2005 and 2006. Since the goal of the present study was to detect and genetically characterize DOBVs in the region, we tested only those animals that perished in the trap naturally. Trapped and tested mammals were identified by an expert taxonomist and all data of the specimens were collected in a computer database (Microsoft Access, 2003). Rodents were dissected and lung tissues were used for hantavirus detection. Total RNA was extracted from lung suspensions with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendation. The RNA was resuspended in 30 μL of RNase-free DEPC-treated water and stored at −70 °C. Reverse transcription (RT) was primed with a virus specific oligonucleotide DOB-S (F) (5′-YTGGYTRAGCATTGTGGTCTA-3′)

* Corresponding author at: Department of Genetics and Molecular Biology, Institute of Biology, University of Pécs, Ifjúság út 6, H-7624 Pécs, Hungary. Tel.: +36 72 503 600; fax: +36 72 503 634.
E-mail address: jakabf@gamma.ttk.pte.hu (F. Jakab).

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Fig. 1. Schematic map of Hungary. The stars indicated the trapping areas and the occurrence of the HFRS patient. The numbers are: (1) Görcsöny, (2) Pécs and (3) Sármellék.

for 1 h at 42 °C, while polymerase chain reaction (PCR) was performed with LightCycler 2.0 instrument using a FastStart MasterPlus SYBR Green I kit (Roche). This reaction was achieved with degenerated gene specific primers DOB-S (F); DOB-S (R) (5′-YTTGGTGGATGGGCCTTTGGT-3′) designed for consensus region of the S-segment of the viral genome and yielding a 433 nt product. Primers were designed with Oligo Explorer 1.2 software and produced by Integrated DNA Tech-

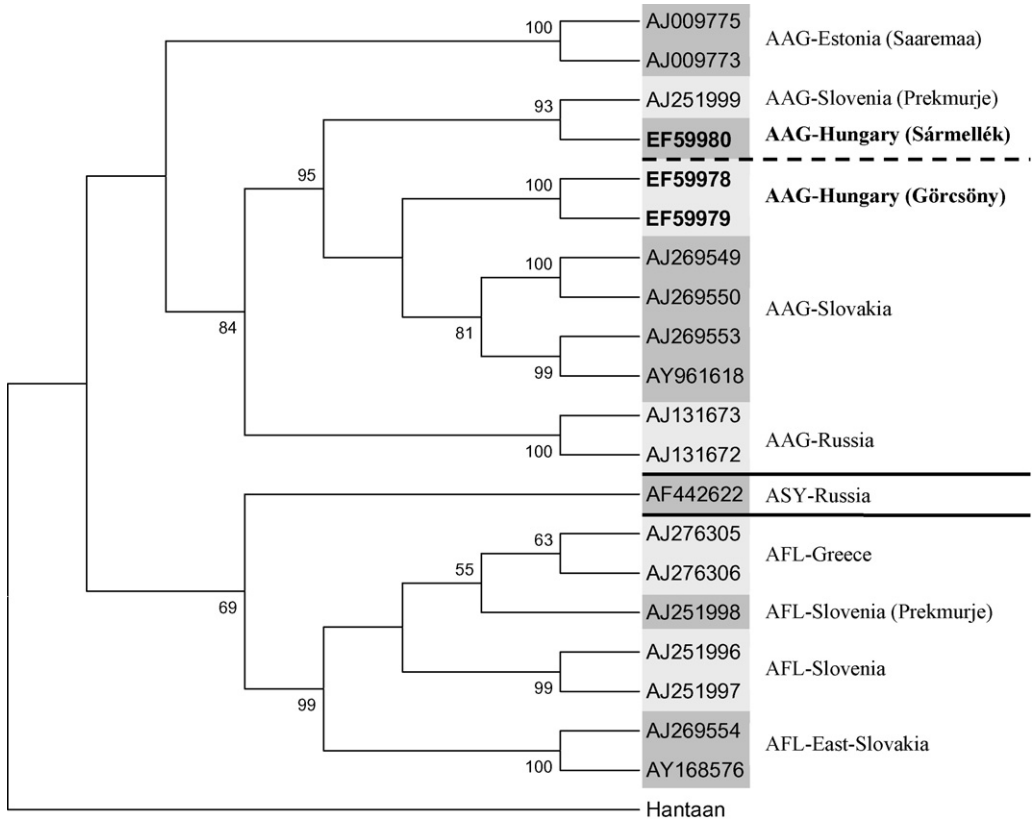

Fig. 2. Based upon a 327 bp long nucleotide sequence of S-segment, a phylogenetic tree was constructed with MEGA v2.1 software using the neighbor-joining algorithm and Kimura 2-parameter model. DOBV sequences isolated from rodents (*Apodemus agrarius*) in Sármellék (DOB/Pecs/77Aa/06-accession number: EF059980) and in Görcsöny (DOB/Pecs/27Aa/06-accession number: EF059978; DOB/Pecs/31Aa/06-accession number: EF059979) are marked in bold face. For other Dobrava hantavirus strains, used the GenBank accession numbers. This is a rooted tree, using Hantaan virus as an outgroup. (*Abbreviations*: AFL, *Apodemus flavicollis*; AAG, *A. agrarius*; ASY, *Apodemus sylvaticus*).

nologies (Coralville, IA, USA). The PCR amplification cycle included an initial denaturation for 10 min at 95 °C, followed by 40 cycles of amplification; denaturation for 10 s at 95 °C, annealing for 10 s at 55 °C, and extension for 10 s at 72 °C. To identify positive samples melting point analysis, as well as standard agarose gel (2%) electrophoresis was performed.

PCR amplicons were purified from the gel using the Wizard SV Gel and PCR Clean-Up system gel extraction kit (Promega, Madison, WI, USA) following the manufacturer's recommendations. Purified PCR products were directly sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit v1.1 (Applied Biosystems, Foster City, CA, USA). The dye-labeled product was analyzed on an ABI Prism 310 DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

Basic sequence manipulation and verification were performed using GeneDoc v2.3 software (http://www.psc.edu/biomed/genedoc/; Nicolas et al., 1997). Nucleotide sequences were aligned by ClustalX v1.8 (http://www.igbmc.u-strasbg.fr/BioInfo; Thompson et al., 1997), which resulted in a consensus nucleic acid sequence alignment of a 327 nucleotides. A phylogenetic tree was constructed from the nucleic acid sequence alignment, using neighbor-joining algorithm with Kimura-2 parameter model of the MEGA v2.1 software (http://www.megasoftware.net; Devereux et al., 1984) using Hantaan virus as an outgroup. The algorithm was replicated 1000 times. Sequences of the Hungarian DOBV strains described here were deposited to GenBank (accession nos. EF059978 to EF059980).

In the present experiment, 22 *Apodemus* mice (*A. agrarius* $n = 11$, *A. flavicollis* $n = 10$ and *A. sylvaticus* $n = 1$) were tested for the presence of DOBV. Of the 22 *Apodemus* rodents, three *A. agrarius* were positive for DOBV by the method described. Two infected animals were captured in the Görcsöny area (southern part of the Transdanubian region) while one virus positive rodent was collected in the Sármellék area (western part of the Transdanubian region); (Fig. 1). Phylogenetic analyses of the 327 base pairs genome fragment (Fig. 2) showed that Hungarian DOBVs were most closely related to Slovenian hantavirus strains. DOBV strain DOB/Pecs/77Aa/06 (EF059980) detected in Sármellék (close to the Slovenian border) was most closely related (97% homology, phylogenetic distance: 0.025) to those viruses isolated from *A. agrarius* mice in Prekmurje (Slovenia); (Avsic-Zupanc et al., 2000). DOBV strains DOB/Pecs/27Aa/06 (EF059978) and DOB/Pecs/31Aa/06 (EF059979) from the Görcsöny location were also closer to the Slovenian hantavirus strains (homology: 94%, phylogenetic distances: 0.061–0.062). Viruses detected in the southern part of the region were slightly different from those viruses originated from the western part of the Transdanubian region. The homology and phylogenetic distance between the Hungarian DOBV strains from different locations were 94% and 0.062 (Table 1). In the phylogenetic tree, three branches of the virus could be distinguished according to their natural rodent host. Unquestionable differences were observed among those viruses detected from *A. agrarius*, *A. flavicollis* or *A. sylvaticus*.

We detected, and molecularly characterized three Hungarian DOBV strains in *A. agrarius* rodents at two locations of the Transdanubian region of Hungary. All three hantavirus strains detected in the region were very similar genetically to those previously detected in Slovenia (Prekmurje). Occurrence of genetically different DOBVs in the region may be an important tool for ecological studies. The need for clinical investigations is supported by a case of severe HFRS caused by DOBV infection in Pécs, close to the Görcsöny trapping site. DOBV was detected in this patient by SYBR Green-based real-time RT-PCR. Sequence data (GenBank accession no. EF028074) established that the patient was infected with DOBV transmitted by *A. flavicollis mice*. In the present investigation, we tested only naturally perished *Apodemus* mice collected during an unrelated ecological study. Based upon our preliminary results, we are planning to expand our trapping area as well as the number of tested rodents using the "kill-trap method". We also intend to assess DOBV-specific seroprevalence in the collected small mammals. Parallel with these small mammalian tests we will survey for new HFRS cases occurring in the region.

Hantaviruses already caused severe outbreaks in Hungary among soldiers during the 1950s (Faludi and Ferenczi, 1995).

Table 1
Main phylogenetic distances among different DOBV strains used in this study

	EF59978	EF59979	EF59980
AAG-Estonia (Saarema)			
AJ009775	0.166	0.166	0.175
AJ009773	0.166	0.166	0.184
AAG-Slovenia (Prekmurje)			
AJ251999	**0.061**	**0.062**	**0.025**
AAG-Slovakia			
AJ269549	0.079	0.079	0.083
AJ269550	0.079	0.079	0.083
AJ269553	0.090	0.083	0.094
AY961618	0.082	0.076	0.087
AAG-Russia			
AJ131673	0.108	0.104	0.105
AJ131672	0.108	0.104	0.105
ASY-Russia			
AF442622	0.185	0.193	0.185
AFL-Greece			
AJ276305	0.163	0.171	0.168
AJ276306	0.163	0.171	0.167
AFL-Slovenia (Prekmurje)			
AJ251998	0.163	0.171	0.168
AFL-Slovenia			
AJ251996	0.159	0.167	0.155
AJ251997	0.155	0.163	0.151
AFL-East-Slovakia			
AJ269554	0.164	0.172	0.160
AY168576	0.164	0.172	0.160
AAG-Hungary (Görcsöny)			
EF59978	–	**0.006**	**0.061**
EF59979	–	–	**0.062**
AAG-Hungary (Sármellék)			
EF59980	–	–	–

The smallest distances between the Hungarian and Slovenian DOBV strains detected in *Apodemus agrarius* mice are marked in bold.

Although the virus had long been present in the country, serological tests from clinical samples were started from the 1980s only. Since then at least 20–25 positive cases become identified annually. This number is probably underestimated because of low awareness of the disease. Nucleic acid of DOBV was detected previously in Hungary by standard RT-PCR (Scharninghausen et al., 1999; Ferenczi et al., 2005).

In this short study, we provided comprehensive molecular data describing the occurrence of DOBVs in the Transdanubian region of Hungary. We also used a new molecular method (SYBR Green-based real-time RT-PCR) to detect these DOBVs in animal tissue using newly designed virus-specific primers. Based our new data from the region we concluded that extended reservoir studies would be necessary in the future.

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