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Keywords:	Hantavirus, Dobrava/Belgrade virus, Slovakia, Czech Republic

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Table 1. Detection of Hantaan virus (HTNV) and Puumala virus (PUUV) reactive antibodies in patients at different time points using commercial ELISA. N: negative result.

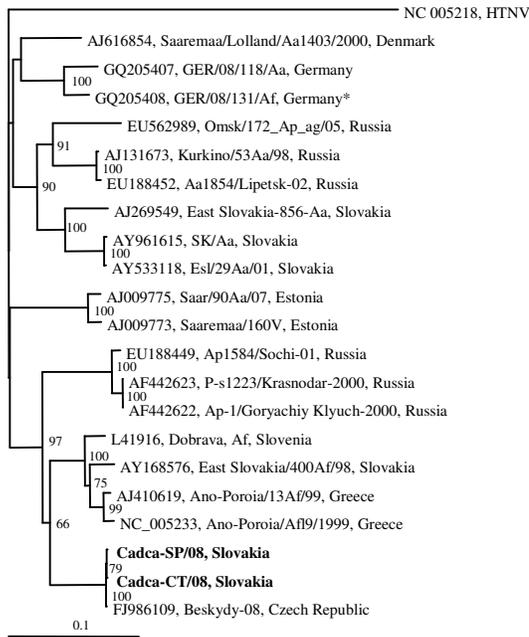
	Day of disease	Index value in HTNV ELISA		Index value in PUUV ELISA	
		IgG	IgM	IgG	IgM
		Patient CT	7	3.21	4.05
	76	2.41	1.46	N	N
Patient SP	13	3.27	6.08	1.28	N
	43	3.39	5.63	1.49	N
	85	3.57	1.49	2.14	N

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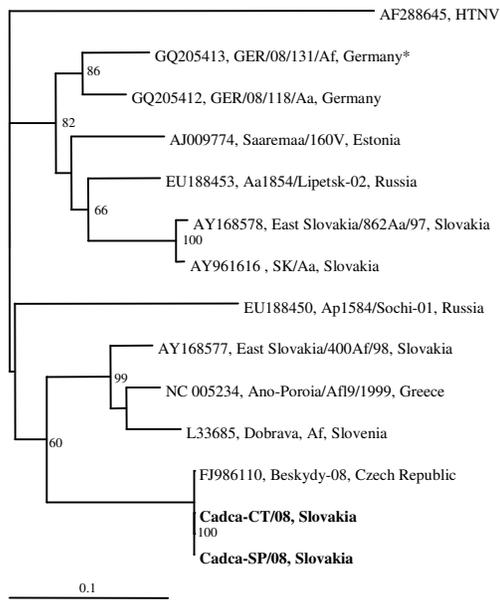
Peer Review

Figure 2A



view

Figure 2B



view

## Hantavirus infection during a stay in a mountain hut in northern Slovakia

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**ABSTRACT**

Hantaviruses in Europe cause human hemorrhagic fever with renal syndrome (HFRS) with various degree of severity. The most severe form is caused by the Dobrava/Belgrade virus (DOBV), associated with the rodent *Apodemus flavicollis*. During the last decade cases of infection caused by DOBV have been reported in Central Europe. The present study is a report on two Czech patients with severe HFRS who were infected during their stay in a mountain hut in Northern Slovakia. The two patients, combined with a third case observed in the same year in a nearby village in the Czech Republic, suggest that this region in Central Europe has to be considered as endemic for HFRS.

**Key words:** Hantavirus, *Dobrava/Belgrade virus*, Slovakia, Czech Republic

## INTRODUCTION

Hantaviruses (genus *Hantavirus*, family *Bunyaviridae*) are enveloped, single-stranded RNA viruses with three-segmented genome, causing to humans hemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus pulmonary syndrome (HPS) in the Americas [Jonsson et al., 2010]. Humans are infected mainly by inhalation of infected rodents' excreta. Five hantavirus genotypes associated with rodents are known to circulate in Europe: *Puumala virus* (PUUV), *Dobrava/Belgrade virus* (DOBV), *Saaremaa virus* (SAAV), *Tula virus* (TUUV) and *Seoul virus* (SEOV). The most pathogenic is DOBV, carried by *Apodemus flavicollis*, with fatality rate up to 10% [Avsic-Zupanc et al., 1999; Papa and Antoniadis, 2001].

Since the first report of hantavirus infections in Breclav, a Czech town in the South Moravian region [Kobzik and Danes, 1992], only a limited number of HFRS cases have been detected in the Czech Republic [Heyman and Vaheri, 2008]. In August 2008, a 15-year boy was infected in Ostravice village, close to the borders of the Czech Republic with Slovakia and Poland, and presented a severe form of HFRS; the patient was hospitalized in the intensive care unit and recovered after 23 days in hospital [Papa et al., 2010]. Application of molecular methods showed that the causative agent was DOBV (strain Beskydy/08), with sequences clustering with respective ones obtained from tissues of *A. flavicollis* trapped in Slovakia, Greece and Slovenia [Papa et al., 2009]. Two severe HFRS cases in Czech patients were noted after staying in a mountain hut in Slovakia are described.

## PATIENTS AND METHODS

On November 29, 2008, a group of 15 Czech young males had a party in a mountain hut in Zakopcie village, near Cadca town, in North Slovakia, near the border with the Czech Republic (49°32'19.077''N, 18°23'29.678''E). The place is 30 km from the Ostravice village in Czech Republic, where a case caused by DOBV was observed in the summer of 2008 [Papa et al., 2010] (Figure 1).

### *Case 1.*

The owner of the hut, a 29-year old hitherto healthy male (patient CT) resident of Bohumín, a small town in the southeastern part of the Czech Republic, went to the hut one day earlier than the rest of the group in order to clean the place. Thirteen days later (December, 10, day 1), he suddenly presented high fever (39°C) accompanied by headache, diarrhea, vomiting, and lower back and abdominal pain. He received antipyretics according to his physician recommendations. However, his condition deteriorated and on December 15 (day 6), he was admitted to the Department of Internal Diseases in the regional hospital in Bohumín. Three days later, he presented signs of gut and skin bleeding, and the patient developed renal failure with proteinuria, hematuria and hypertension. Hemodialysis was not applied.

The patient presented leucopenia  $3.5 \times 10^9/l$  (normal range  $4-10 \times 10^9/l$ ), thrombocytopenia  $18 \times 10^9/l$  (normal range  $150-400 \times 10^9/l$ ), and elevated liver enzymes. Serum urea increased to 21.0 mmol/l (normal range 2.8-7.2 mmol/l), creatinine to 245  $\mu\text{mol/l}$  (normal range 62-115  $\mu\text{mol/l}$ ), CRP to 45 mg/l (normal range 0-10 mg/l). He presented high values of D-dimers (maximum of 18.25 mg/l, normal range 0-0.2 mg/l);

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3 other coagulation parameters were slightly affected. Renal biopsy was suggestive of  
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5 progressive acute glomerulonephritis, while the thrombocytopenia was considered as  
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7 immune-associated. As his condition deteriorated rapidly, on day 10 he was transferred to  
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9 the Intensive Care Unit of the Department of Internal Diseases in the University Hospital  
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11 in Ostrava. His clinical condition, the renal and liver function, and the laboratory findings  
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13 returned subsequently to normal and he was discharged from the hospital on day 19, with  
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15 the diagnosis of hemolytic uremic syndrome. On January 22, 2009, (day 43) during a  
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17 follow-up examination in a mobile medical facility, a hantavirus infection was suspected.  
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#### 24 *Case 2*

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26 On December 26, 2008, 28 days after the overnight in the mountain hut, a second  
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28 member of the group, a 27-year old hitherto healthy male (SP), developed a sudden high  
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30 fever (40°C) with profuse diarrhoea, vomiting, headache and vertigo. On day 4 the  
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32 patient collapsed, and he was admitted to the Department of Internal Diseases of the  
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34 regional hospital in Bohumín. His condition deteriorated rapidly with hypovolemic and  
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36 haemorrhagic shock leading to multiorgan failure with predominant renal failure and  
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38 anuria. His condition was complicated by peripheral edema of the lung. The patient had  
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40 disseminated intravascular coagulation with heavy bleeding from the needle puncture  
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42 sites, and then, ongoing bleeding into the adrenal and retroperitoneal space, prompting  
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44 urgent transfer to the Department of Anaesthesiology and Resuscitation in the University  
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46 Hospital in Ostrava. High levels of procalcitonin (88.6 µg/l, normal range 0-0.5 µg/l)  
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48 were observed, however, with no evidence of bacterial infection. Hemodialysis and  
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arteficial ventilation with tracheostomy were applied. The patient remained comatose for  
several weeks.

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Laboratory findings included leucocytosis  $24.4 \times 10^9/l$  (normal range  $4-10 \times 10^9/l$ ),  
thrombocytopenia  $3 \times 10^9/l$  (normal range  $150-400 \times 10^9/l$ ), elevated liver enzymes –  
ALT  $5.97 \mu\text{kat/l}$  (normal range  $0.15-0.75 \mu\text{kat/l}$ ), AST  $8.44 \mu\text{kat/l}$  (normal range  $0.15-$   
 $0.85 \mu\text{kat/l}$ ). Serum urea increased to  $20.3 \text{ mmol/l}$  (normal range  $2.8-7.2 \text{ mmol/l}$ ),  
creatinine to  $474 \mu\text{mol/l}$  (normal range  $62-115 \mu\text{mol/l}$ ). The maximum value of CRP was  
127 mg/l (normal range  $0-10 \text{ mg/l}$ ). He revealed extreme values of D-dimers with the  
maximum value  $24.67 \text{ mg/l}$  (normal range  $0-0.2 \text{ mg/l}$ ); other coagulation parameters  
were also affected - prothrombin time 26 s (normal range 11-16 s), and activated partial  
thromboplastin time 80.9 s (normal range 25-35 s).

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The condition of the patient was complicated further by lifethreatening nosocomial  
infections caused by methicillin-resistant *Staphylococcus aureus* and *Candida* spp. He  
was discharged after 68 days in hospital, with most of the time in the Department of  
Anaesthesiology and Resuscitation.

### Serology

Serum samples taken on days 7 and 76 from patient CT and on days 13, 43 and 85 from  
patient SP were tested for IgG and IgM antibodies to Hantaan virus (HTNV) and PUUV  
using ELISA (PROGEN Biotechnik, Heidelberg, Germany). IgM and IgG serology  
results are considered positive when the index value (optical density divided by the cut  
off value) is  $\geq 2$  and  $\geq 1.5$  respectively.

### *Extraction of viral RNA and polymerase chain reactions*

Viral RNA was extracted from the earliest available serum samples (taken on day 7 from patient CT and on day 13 from patient SP) using the QIAamp Viral RNA kit (Qiagen, Hilden, Germany) and two RT-nested PCRs were performed using two sets of primers designed to detect hantaviruses associated with rodents of the Murinae subfamily (HTN, DOB, SEO). One primer set (MS120C - MS1170R for the first round and MS364C - MS963R for the second round) was used for the amplification of a 590-bp fragment of the N coding region, and a second primer set (MM1470C - MM2029R for the first round and MM1674C - MM1990R for the second round) was used for the amplification of a 317-bp fragment of partial G1 coding region [Papa et al., 1998; Papa et al., 2009].

### *Sequencing and phylogenetic analysis*

Sequencing of the PCR products was performed in an ABI 3110 genetic analyzer (Applied Biosystems, Foster City, CA). Sequences were aligned by CLUSTAL W, while phylogenetic trees based on S and M segment sequences were constructed by Neighbor-joining method using the modules DNAdist, Neighbor and Consense from the PHYLIP software [Felsenstein, 1993]

## RESULTS

IgM and IgG antibodies against HTNV were detected in all serum samples tested, while antibodies against PUUV were either not detected or the titers were low (Table 1).

Serology results suggested that the causative agent was a hantavirus closer antigenically to HTNV. Products of the expected size were obtained by both RT-nested PCRs, while sequencing analysis confirmed that the causative agent was DOBV. The two DOBV sequences (Cadca-CT/08 and Cadca-SP/08) were identical. The closest strain genetically was the Czech strain Beskydy/08, presenting one nucleotide difference in S segment, and no difference in the M segment. In both S and M segment phylogenetic trees, the sequences from both patients and those of the Czech strain (Beskydy/08, Cadca-CT/08, Cadca-SP/08) comprise a distinct subclade in the clade containing DOBV sequences associated with *A. flavicollis* (Figure 2A and 2B). In the S segment, Slovakian strain differs by 8.7% and 8.6% from DOBV strains isolated from *A. flavicollis* in Greece and Slovenia respectively, and by 9.5% from strain 400Af/98 from East Slovakia, while in the M segment the Slovakian strain differs by 15.5% from the strain 400Af/98, and by 16.7% and 18.6% from strains isolated in Greece and Slovenia respectively.

## DISCUSSION

DOBV is highly endemic in the Balkan peninsula, where cases are observed usually in the summer time [Avsic-Zupanc et al., 1999; Papa and Antoniadis, 2001]. In Central and Northern Europe HFRS cases are caused mainly by PUUV [Heyman et al., 2009; Vapalahti et al., 2003]. However, during the last decade, cases caused by DOBV have been reported in Slovakia, Germany, Poland and the Czech Republic [Klempa et al., 2004; Nowakowska et al., 2009; Papa et al., 2010; Sibold et al., 2001]. The patients of the

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2  
3 present study were infected in November 2008 in Slovakia, in an area 30 km far from  
4 Ostravice village (South-eastern Czech Republic), where an HFRS case was observed in  
5 August 2008, suggesting that this area of Central Europe is an endemic focus for HFRS.  
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8 The occurrence of the cases in winter could be explained by the fact that rodents entered  
9 the hut trying to be protected from the cold. Patient CT presented the disease earlier than  
10 patient SP (incubation time 13 days for patient CT versus 28 days for patient SP), most  
11 probably because CT inhaled the largest quantity of aerosolized excreta, as he was the  
12 first who entered the hut, he cleaned the place and he stayed more time there than the rest  
13 group. Two other members of the group tested negative for the presence of IgG and IgM  
14 antibodies, while the rest refused to be tested. Different factors, including variations in  
15 immune response might explain why only patient SP (who stayed the same time as the  
16 rest) acquitted the disease. Although the incubation time of patient SP was longer than  
17 that of patient CT, his course of the disease was much more severe, accompanied by  
18 heavy hemorrhages, and more affected hematological and biochemical parameters.  
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20 Cytokines and chemokines might be responsible for the different response, which  
21 resulted in prolongation of the hospitalization time and further acquisition of nosocomial  
22 infections. Preliminary studies have shown that a large number of cytokines and  
23 chemokines play a catalytic role in the course and the outcome of HFRS; among them  
24 PDGF, RANTES, IP-10, MIP-1b, IL-1ra, IL-9, GM-CSF, IFN-g, eotaxin, and MCP-1 are  
25 the most important [Tsergouli et al., 2010].  
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50 The Slovakian and the Czech sequences cluster with DOBV strains associated with *A.*  
51 *flavicollis*, and this is in accordance that this virus causes more severe disease than the  
52 other hantaviruses in Europe. Studies in rodents in the area will help the identification of  
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3 the exact host of these viruses. It has to be mentioned that DOBV spill-over events have  
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5 been reported in rodents [Schlegel et al., 2009; Weidmann et al., 2005].  
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8 A geographical clustering of sequences has been reported among hantaviruses [Papa et  
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10 al., 1998; Weidmann et al., 2005]. The strains of the present study differ greatly from  
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12 those detected in East Slovakia, and, more interestingly, in the S segment they are closer  
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14 genetically to Greek DOBV strains than to East Slovakian ones. . It should be noted that  
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16 there are high mountains between Cadca region, the place where patients of the present  
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18 study were infected, and Kosice, the place where strain East Slovakia/400Af/98 was  
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20 isolated. Full genome sequencing of the strains will reveal whether recombination or  
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22 reassortment events took place.  
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27 The results of the present study demonstrate that severe DOBV cases are observed in the  
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29 area where the Czech Republic borders with Slovakia and Poland. Further studies are  
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31 needed to identify the exact host(s) of the hantavirus strains circulating in that area, while  
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33 full genome sequence determination will give insight into the genetic characterization of  
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35 the strains. Apart from this, clinicians have to include HFRS in the differential diagnosis  
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37 of febrile cases among persons who spend time in the countryside in this region,  
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39 especially when they present thrombocytopenia and acute renal insufficiency.  
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**REFERENCES**

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- Avsic-Zupanc T, Petrovec M, Furlan P, Kaps R, Elgh F, Lundkvist A. 1999. Hemorrhagic fever with renal syndrome in the Dolenjska region of Slovenia - a 10-year survey. *Clin Infect Dis* 28:860-865.
- Felsenstein J. 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle.
- Heyman P, Vaheri A. 2008. Situation of hantavirus infections and haemorrhagic fever with renal syndrome in European countries as of December 2006. *Euro Surveill* 13: pii: 18925.
- Heyman P, Vaheri A, Lundkvist A, Avsic-Zupanc T. 2009. Hantavirus infections in Europe: from virus carriers to a major public-health problem. *Expert Rev Anti Infect Ther* 7:205-217.
- Jonsson CB, Figueiredo LT, Vapalahti O. 2010. A global perspective on hantavirus ecology, epidemiology, and disease. *Clin Microbiol Rev* 23:412-441.
- Klempa B, Schutt M, Auste B, Labuda M, Ulrich R, Meisel H, Kruger DH. 2004. First molecular identification of human Dobrava virus infection in central Europe. *J Clin Microbiol* 42:1322-1325.
- Kobzik J, Danes L. 1992. [Laboratory-confirmed cases of hemorrhagic fever with renal syndrome which occurred in Breclav 1989-1990]. *Cesk Epidemiol Mikrobiol Imunol* 41:65-68.
- Nowakowska A, Heyman P, Knap JP, Burzynski W, Witas M. 2009. The first established focus of hantavirus infection in Poland, 2007. *Ann Agric Environ Med* 16:79-85.

- 1  
2  
3 Papa A, Antoniadis A. 2001. Hantavirus infections in Greece - an update. Eur J  
4  
5 Epidemiol 17:189-194.  
6  
7  
8 Papa A, Johnson AM, Stockton PC, Bowen MD, Spiropoulou CF, Alexiou-Daniel S,  
9  
10 Ksiazek TG, Nichol ST, Antoniadis A. 1998. Retrospective serological and  
11  
12 genetic study of the distribution of hantaviruses in Greece. J Med Virol 55:321-  
13  
14 327.  
15  
16  
17 Papa A, Zelena H, Barnetova D, Petrousova L. 2010. Genetic detection of  
18  
19 Dobrava/Belgrade virus in a Czech patient with Haemorrhagic fever with renal  
20  
21 syndrome. Clin Microbiol Infect. 16:1187-1190.  
22  
23  
24 Schlegel M, Klempa B, Auste B, Bemann M, Schmidt-Chanasit J, Buchner T,  
25  
26 Groschup MH, Meier M, Balkema-Buschmann A, Zoller H, Kruger DH, Ulrich  
27  
28 RG. 2009. Dobrava-Belgrade virus spillover infections, Germany. Emerg Infect  
29  
30 Dis 15:2017-2020.  
31  
32  
33 Sibold C, Ulrich R, Labuda M, Lundkvist A, Martens H, Schutt M, Gerke P, Leitmeyer  
34  
35 K, Meisel H, Kruger DH. 2001. Dobrava hantavirus causes hemorrhagic fever  
36  
37 with renal syndrome in Central Europe and is carried by two different *Apodemus*  
38  
39 mice species. J Med Virol 63:158-167.  
40  
41  
42  
43 Tsergouli K, Papadimitriou E, Gavana E, Papa A. 2010. Cytokine levels in hemorrhagic  
44  
45 fever with renal syndrome caused by Dobrava-Belgrade virus. In: VIII  
46  
47 International Conference on HFRS, HPS and Hantaviruses. Athens, Greece. p  
48  
49 206.  
50  
51  
52  
53 Vapalahti O, Mustonen J, Lundkvist A, Henttonen H, Plyusnin A, Vaheri A. 2003.  
54  
55 Hantavirus infections in Europe. Lancet Infect Dis 3:653-661.  
56  
57  
58  
59  
60

Weidmann M, Schmidt P, Vackova M, Krivanec K, Munclinger P, Hufert FT. 2005.

Identification of genetic evidence for Dobrava virus spillover in rodents by nested reverse transcription (RT)-PCR and TaqMan RT-PCR. J Clin Microbiol 43:808-812.

For Peer Review

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3 Figure legends  
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6 Figure 1. Map showing the site in N. Slovakia where patients of the present study got  
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8 infected; site in the Czech Republic where a patient of a previous study got infected is  
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10 also shown.  
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13 Figure 2. Phylogenetic trees based on a 590-bp fragment of the S RNA segment (A) and a 317-  
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15 bp fragment of the M RNA segment (B) of hantaviruses. HTNV was used as outgroup. The  
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17 numbers at the nodes indicate percentage bootstrap replicates of 100; values below 60%  
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19 are not shown. Horizontal distances are proportional to the nucleotide differences. The  
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21 scale bar indicates 10% nucleotide sequence divergence. Sequences in the tree are  
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23 indicated as GenBank accession number, strain name, country. Strains of the present  
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25 study are shown in bold. Af: *Apodemus flavicollis*, Aa: *Apodemus agrarius*, Ap:  
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27 *Apodemus ponticus*. \*The sequence GER/09/131/Af originated from a DOBV-Aa  
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29 spillover infected *A. flavicollis* rodent.  
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