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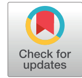
Molecular characterization of Dobrava-Belgrade hantavirus in Serbia, 2007–2011



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ABSTRACT

Background: Hantaviruses are etiological agents of emerging zoonotic diseases worldwide, including hemorrhagic fever with renal syndrome (HFRS). A number of hantavirus species is known to be present in Europe. In Serbia, existing data on hantavirus presence and prevalence rely in serological findings. In this study, molecular analysis was performed in order to characterize HFRS causing hantaviruses in Serbia.
Methods: Sixty four serum samples of HFRS cases, previously found seropositive to anti-hantaviral antibodies, were included in the study. Partial hantaviral L and S segments were PCR amplified producing 390nt and 598nt amplicons, respectively, in parallel with human beta-actin mRNA as external reverse transcription positive control. Hantavirus specific PCR products were DNA sequenced in both direction and the obtained sequences phylogenetically confirmed and analyzed.
Results: PCR detection of hantavirus L and S genome segments was positive in 18/64 and 11/64 tested samples, respectively. Positive PCR results involved samples obtained from different locations, mostly from central and southern parts of Serbia. All the obtained sequences were identified as Dobrava-Belgrade virus (DOBV). In the phylogenetic analysis sequences from Serbia tended to cluster in distinctive, geographically related clusters.
Conclusions: Our findings indicate DOBV as the main HFRS causing hantavirus in Serbia, the site of its initial isolation.

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Introduction

Hantaviruses have gained worldwide attention as etiological agents of emerging zoonotic diseases, namely hemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus cardiopulmonary syndrome (HCPS) in Americas, with fatality rates ranging from <10% up to 60% [1]. They are members of the family *Hantaviridae*, order *Bunyavirales*, with three-segmented (large (L), medium (M) and small (S)) RNA genome. [2]. Hantavirus reservoir species include rodents, members of different subfamilies comprising *Sigmodontinae*, *Arvicolinae*, *Murinae* and *Neotominae,* as well as insectivores and bats [3,4]. In Europe, at least four hantavirus species are found: Dobrava-Belgrade (DOBV), Seoul (SEOV), Puumala (PUUV) and Tula (TULV), primarily, but not exclusively, associated with *Apodemus flavicollis*, *Rattus norvegicus, Myodes glareolus* and *Microtus arvalis*, respectively [2]. PUUV is widespread throughout Europe while DOBV presence is significant in Central and South-East Europe, especially the Balkans and parts of Russia.

Serbia is central/southeast European country, situated in Southern Pannonian Plain and the central Balkans. HFRS is known to be present in Serbia since mid-20th century, whereas hantavirus isolates from human cases in Serbia, dating from 1988, were the very first ones from the Balkan region [5]. However, up to now very few molecular genetic studies of hantavirus isolates from Serbia, have been reported, in particular from cases of human infection [1].

Methods

Serum samples of HFRS cases, previously determined seropositive for hantaviruses at the Serbian National Reference Laboratory for Arboviruses from January 2007 to October 2011, were included

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Fig. 1. Map of Serbia showing sites of host infection for the analyzed DOBV strains.
The figure illustrates sites of origin of all NCBI DOBV sequences deposited from Serbia until December 2016; sequence names are derived from the accession number and year of detection; human/rodent source material is depicted by the relevant symbol.

in the study. In this time period, a total of 699 samples of clinically suspective HFRS cases had been submitted to the Serbian National Reference Laboratory for Arboviruses for serological testing, whereas 117/699 sera had been found positive for either IgM or/and IgG for human hantaviruses [6]. Sixty-four of these 117 sera were available for retrospective PCR analysis within this study. After total RNA extraction by QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) reverse transcription was performed using One Step RNA PCR Kit (Qiagen, Hilden, Germany), followed by nested PCR testing with PCR Core Kit (Qiagen, Hilden, Germany). Protocol included sets of degenerate primers for hantavirus L and S segments, producing 390nt and 598nt amplicons, respectively [7,8]. Amplification of human beta-actin mRNA (primers sequences: 5′- CATGTGCAAGGCCGGCTTCG-3′ and 5′- GAAGGTGTGGTGCCAGATTT-3′) was used as external reverse transcription positive control. Obtained hantavirus amplicons were sequenced in both directions by dye-terminator sequencing on ABI 310 automated DNA sequencer (Applied Biosystem, Foster City, CA, USA).

Initial identification of the obtained sequences was done using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and further phylogenetically confirmed based on clustering in relation to hantaviral reference sequences. Multiple nucleotide sequence alignments were created using CLUSTAL W, as implemented in MEGA 6 software [9].

Sequence analysis was processed in Paup 4.0, MrBayes and Beast softwares using neighbour-joining, maximum likelihood and minimum evolution methods [10–12]. To characterize genetic diversity of the viral strains isolated from Serbian patients and assess their phylogenetic relationships, the obtained sequences were compared

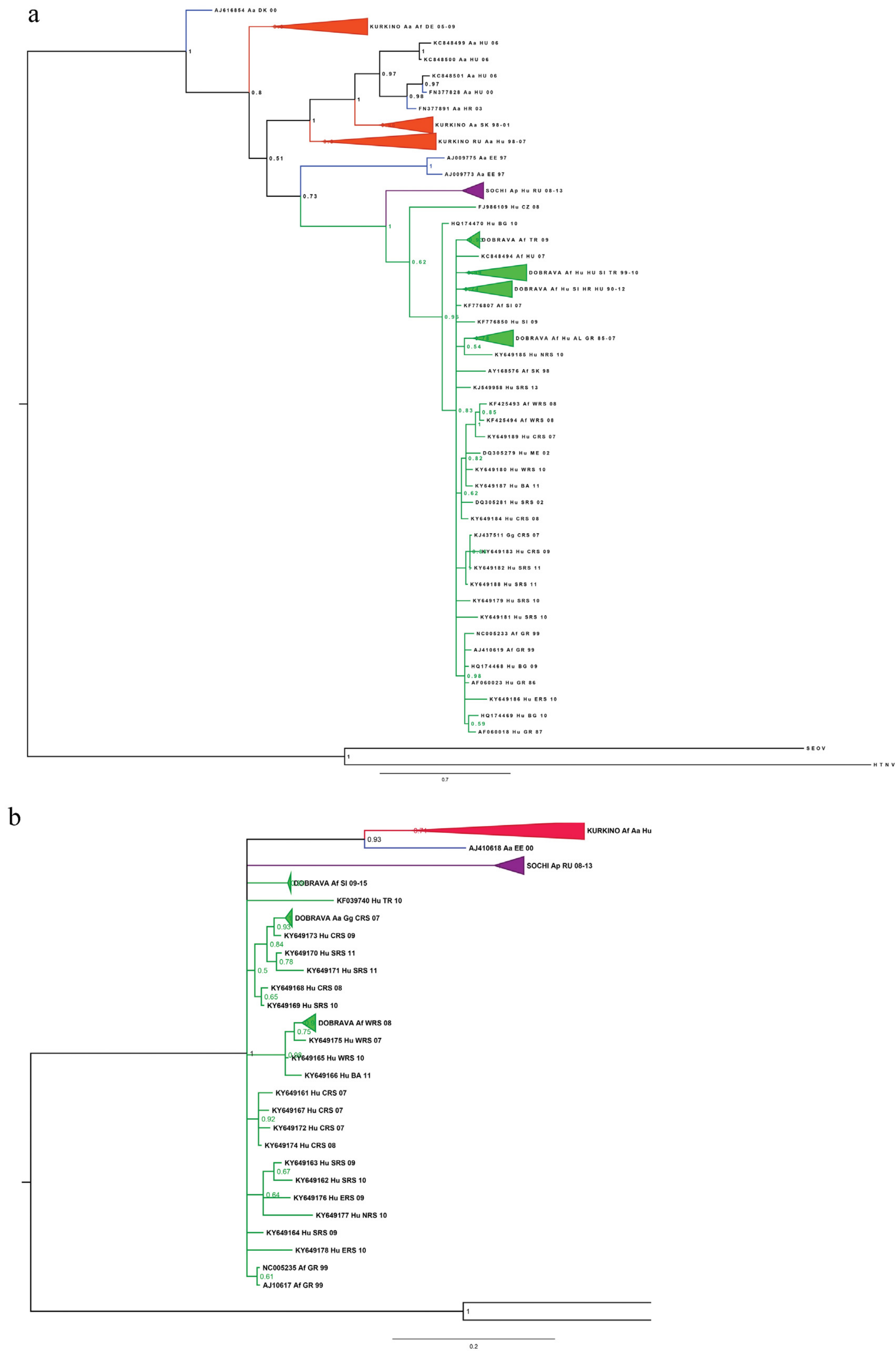

Fig. 2. Phylogenetic trees of the analyzed partial S (a) and partial L (b) segments.
Phylogenetic trees were inferred using the Bayesian methods. Numbers on branches indicate posterior node probabilities. Scale bar represents number of nucleotide substitutions per site. Twenty-nine identical sequences were excluded from the analyses. DOBV genotypes Saaremaa, Dobrava, Kurkino and Sochi shown in blue, green, red and purple, respectively; NC005236 and NC005238 (Seoul hantavirus), NC005218 and NC005222 (Hantaan hantavirus) were used as outgroups.

to all corresponding DOBV L and S segment sequences present in the GenBank database until December 2016 (Supplementary Table 1), resulting in two alignments, of 37 L segment sequences (3023nt–3282nt) and 180 S segment sequences (362nt–855nt), including the corresponding outgroups. Nucleotide substitution rates were calculated by Relaxed Lognormal clock model integrated in Beast v.1.8.3. Evolutionary pressure was assessed using HyPhy software package implemented by the Datamonkey web-based facility (http://www.datamonkey.org).

Results

PCR detection of hantavirus L and S genome segments was positive in 18/64 and 11/64 tested samples, respectively. Positive PCR results involved samples obtained from different locations, mostly from central and southern parts of Serbia (Fig. 1). All samples with positive PCR amplification of the S segment were also found positive for the L segment. All the obtained sequences were identified as DOBV and submitted to the GenBank (accession numbers listed in the Supplementary Table 1).

S segment based phylogenetic tree contained 180 DOBV sequences from human and rodent samples and included 17 unique sequences from Serbia: 11 sequences generated during the current study and 6 sequences obtained from the database. General topology of the tree showed 4 clusters corresponding to presumed DOBV genotypes: Dobrava, Kurkino, Sochi and Saaremaa (Fig. 2). All Serbian strains clustered within the Dobrava genotype. Majority of Serbian strains (15/17) clustered separately, without intermixing with other strains (Fig. 2A). Within them, 12/17 sequences formed two distinct branches, while three strains formed separate, isolate branches. Two remaining Serbian sequences clustered with Greek, Bulgarian and Albanian strains (Fig. 2A). In the phylogenetic tree based on 37 L segment sequences strains from Serbia clustered in a distinctive branch of Dobrava genotypes with strains from Greece, Slovenia and Turkey (Fig. 2B).

Overall nucleotide diversity found in the partial S segment alignment was in the expected range of 7.17% (SD ± 0.05), with average distance between Serbian strains and all other DOBV samples of 5.84% (SD ± 0.004). Diversity among human strains from Serbia was higher compared to animal isolates (2.23%, SD ± 0.01 vs. 1.79%, SD ± 0.01). In the L segment, the obtained average nucleotide distance was 10.81% (SD ± 0.06), with higher value for Serbian strains (14.09%, SD ± 0.06).

The analysis of partial S segment alignment of 500 nt resulted in substitution rate estimation of 2.30×10^{-4} (SE of mean = 3.64×10^{-6}; 95% HPD interval: $1.32–3.35 \times 10^{-4}$). No evidence of positive selection was detected along the studied alignment, since all nucleotide substitutions were found to result from negative selective pressure, with the overall dN/dS = 0.0224.

Discussion and conclusion

Hantaviruses are endemic in the Balkan region, particularly in Serbia, where sporadic cases and/or outbreaks of HFRS have been repeatedly reported [13]. With emerging pathogens as hantaviruses, knowledge of the local viral distribution in endemic areas is of vital importance for global surveillance, in relation to both prospective preventive measures and potential vaccine development. So far, serological findings in both human cases and in rodent reservoirs, implied circulation of multiple hantaviruses in Serbia, including HFRS-causing DOBV and PUUV [1]. Recently, we have genetically characterized hantaviruses from rodent hosts in Serbia, namely TULV in *Microtus arvalis* and DOBV in *Apodemus* mice and *Glis glis* [14–16]. However, no studies including genetic characterization of hantaviruses in Serbian HFRS patients, existed up to now, except for three strains obtained during an epidemic in 2002, when 128 cases were serologically confirmed in Serbia and Montenegro [1]. Here, we present the first genetic characterization study on a set of 64 hantavirus seropositive samples of HFRS patients from Serbia. Obtained phylogenetic clustering was in line with the defined DOBV genotypes: Dobrava, Kurkino, Sochi and Saaremaa [17]. Notably, all the obtained Serbian sequences were identified as Dobrava-Belgrade virus of Dobrava genotype, in spite of historic serological evidence of the presence of other hantaviruses locally [13]. Serology remains the mainstay of diagnosis of hantavirus infections as the viremia is rather short-lived, however, considerable cross-reactivity between related hantavirus has been described [1]. Hence, in view of the retrospective testing approach, this finding might be influenced by differing duration and level of viremia in different hantavirus infections — with, generally, higher viremia more often reached in DOBV, compared with PUUV infection [18]. Nevertheless, our results may be considered to imply that DOBV is the main HFRS causing hantavirus in Serbia. This finding highlights the need for enhanced caution in the clinical management of affected patients and for targeted preventive measures in rodent control. Phylogenetic analysis of the newly acquired sequences of DOBV suggested local geographic-specific clustering, evidenced by phylogenetic analysis in both L and S segment genetic regions, as previously shown for the majority of hantaviruses [1]. Regarding diversity analysis, high substitution rate was found, of the order of 10^{-3} substitutions/site/year, as typically described for RNA viruses [19]. However, in contrast to high substitution rate, all nucleotide substitutions were found to be under negative selective pressure. Selection pressure analysis was performed on partial S segment alignment, not allowing insight into the evolution of the whole segment. However, as shown for other viruses, functional aspects of viral proteins are those that often significantly effect on selection pressure, limiting fixation of new mutations [20].

In conclusion, presented findings of PCR positive DOBV samples indicate that DOBV is the main HFRS causing hantavirus in Serbia, the site of its initial isolation. Further studies are needed to achieve better insight into the molecular epidemiology of DOBV related to human cases in Serbia and in the region.

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Competing interests

None declared

Ethical approval

Not required

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jiph.2019.02.021.

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