UNIVERSITY OF PÉCS

Biological Doctoral School

Detection of Dobrava-Belgrade hantaviruses among *Apodemus* mice using molecular and serological methods

PhD thesis

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INTRODUCTION

Hantaviruses belong to the *Bunyaviridae* family. Virions have globular shape and measure 80-120 mm in diameter. Hantaviruses can be divided into two groups based on their geographic distribution. Old World hantaviruses are widespread in Eurasia and Africa and cause hemorrhagic fever with renal syndrome (HFRS). Their main representatives are Hantaan virus (HTNV) in Asia, while Dobrava-Belgrade (DOBV) and Puumala (PUUV) viruses in Europe. New World hantaviruses are circulating in the Americas and cause hantavirus cardiopulmonary syndrome (HCPS); with Sin Nombre virus (SNV) being the most important pathogen.

Hantaviruses have a tripartite, negative-sense single-stranded RNA genome of about 12 kilobases in size. The S, (1.7-2.0 kb), M, (3.6-3.7 kb) and L segments (6.5 kb) encode the nucleocapsid protein (NP), the glycoproteins (G_N, G_C) and the RNA-dependent RNA polymerase, respectively. The latter protein has endonuclease, transcriptase, replicase and RNA-helicase activity. NPs and RNA segments together build up the ribonucleoproteins, which are surrounded by a lipid bilayer, incorporating the glycoprotein heterodimers. In infected cells, only the capsid-covered viral RNA is recognized by the RNA-dependent RNA polymerase, hence the presence of capsid proteins is essential for viral transcription and replication. Glycoproteins maintain virion attachment to the target cell receptors, and also mark their intracellular assembly sites.

The main carriers of hantaviruses are rodents belonging to the *Murinae, Arvicolinae* and *Sigmodontinae* taxons. In the last decade, hantaviruses could also be detected in different shrew (family *Soricide*) and mole (family *Talpidae*) species of the order *Soricomorpha.* Bat-transmitted (order *Chiroptera*) hantaviruses are considered the most recent representatives. Hantaviruses are transmitted by vaporization of the body fluids of infected rodents (saliva, urine and stool), or via biting. Rodents shed the viruses often through their lifetime. Humans are considered to be a dead-end for hantaviruses, however, in the case of Andes virus (ANDV) in Argentina, human-to-human transmission was proven.

Old world hantaviruses cause HFRS or neprhopathia epidemica (NE), a milder from of the syndrome with renal involvement. Diagnosis is difficult due to early stage hantavirus infections being aspecific and often showing flu-like symptoms. Only one-third of patients have specific symptoms such as renal failure and hemorrhagic manifestations. Case fatality

rate for infections with severe HFRS is around 5-10%, while it is only 0.1% for the milder NE.

In Europe, DOBV, PUUV, Saaremaa (SAAV), Tula (TULV) and Seoul (SEOV) viruses are present. PUUV is the most widespread and causes the most human infections, since its host, the bak vole (*Myodes glareolus*) can be found in most parts of Europe. The second main infectious agent is DOBV, responsible for the severe HFRS cases. It has three clades according to the rodent host species. DOBV-Af, carried by the yellow-necked mouse (*Apodemus flavicollis,* Af), which is a natural host for hantaviruses throughout whole Europe; DOBV-Aa, which is carried by striped field mice (*Apodemus agraius*, Aa), living in Central Europe, and DOBV-Ap, carried by Back Sea field mice (*Apodemus ponticus*, Ap) in the Caucasus. Southern Europe and especially the Balkans are dominated by DOBV-Af infections. DOBV-Aa infections are characteristic for Russia. The fierce debate regarding the taxonomy of DOBV has started a couple of years ago. On the Estonian island of Saaremaa, a virus with high genetic similarity to DOBV was detected in *A. agrarius,* later named SAAV. Initially, this pathogen was considered to be a genetic variant of DOBV. Later, the International Committee on Taxonomy of Viruses (ICTV) declared SAAV to be a separate subtype of hantaviruses. However, some researchers suppose, that not all viruses detected in *A. agrarius* in Central Europe are SAAV.

AIMS OF THE STUDY

1. Production of recombinant DOBV capsid antigen utilizing an *Escherichia coli* expression system,
2. Development of a new, recombinant antigen-based ELISA test, suitable for both research and diagnostic applications,
3. The survey of prevalence and abundance of rodent-transmitted hantavirus strains in Hungary and near the Croatian-Hungarian border areas,
4. Identification of novel hantavirus strains,
5. The carry out of seroepidemiological assays using the produced recombinant antigen
6. The molecular characterization of detected virus strains by phylogenetical and sequential analysis and the identification of evolutional relations and lineages.

MATERIALS AND METHODS

Samples

Our research focused on two rodent species, the yellow-necked field mouse (*Apodemus flavicollis*, Af) and striped field mouse *(Apodemus agrarius*, Aa), both species captured at five Southern-Transdanubian (Pécs-Arpádtető, Gyékényes, Görcsöny, Gyód, Sármellék), and two Croatian (BeliManastir, Gola) locations. Animals that perished in the trap were forwarded for research purposes. Lung tissues and blood were used as a source to extract viral RNA. In addition, we were granted human serum samples from hospitalized patients.

Molecular biological tests, phylogenetic analysis

Viral nucleic acid (RNA) was extracted using TRIzol® (Invitrogen) method according to the manufacturer's recommendations, followed by reverse transcription (RT) using 5' oligonucleotids. For molecular characterization, Qiagen one-step RT-PCR kit was utilized with primers designed to amplify the S-segment coding region of the viral nucleic acid. For sequence analysis, DNA templates obtained from one-step RT-PCR were amplifyed by nested PCR. Sequencing was carried out using ABI Prism 310 sequence analyzer.

Sequence editing and nucleotid aligment was carried out using GeneDocv2.7 and ClustalXv2.0 softwares, respectively. Phylogenetic trees were generated using MEGA v5.0

software, with Maximum Likelihood method and General Time Reversible substitution model (with invariable positions and gamma distribution, GTR+I+G). Number of bootstraps for simulations was 1000, in addition, an ourgroup was also used to achieve more precise results.

Protein-expression and serological assays

We have produced a recombinant viral antigen in a bacterial expression system for use in DOBV serological assays. The coding seuence of the viral nucleocapsid protein was cloned into pET28a+ vector. *Escherichia coli* BL21 Rosetta competent cells were used for transformation. Rosetta cells containing the recombinant plasmid were propagated in LB medium with 30 μg/ml kanamycin and 35 μg/ml chloramphenicol added. Incubation was carried out at 37°C in a shaker at 200 rpm. When reaching log phase (OD_{600} = 0.8-1.0), cells were inducated with 1.0 mM IPTG. Induction was carried out at 15°C for 20 hours (O/N), followed by cell precipitation. Protein was extracted from inclusion bodies under denaturing conditions using chaotropic agents. Protein purfication was carried out on HIS Select®HF NickelAffinityGel, different pH fractions were visualized with SDS-polyacrilamide gel electrophoresis with Comassie Blue staining.

Viral nucleocapsid antigen was utilized by an in-house development ELISA assay optimized for both animal and human samples. Western blot analyses were also carrid out in order to test recombinant protein activity and to confirm results.

RESULTS AND DISCUSSION

Molecular tests

Our investigation started in 2009 with the aims of molecular testing of DOBV and mapping its genetic variablity. Further molecular biological, immunological and epidemiological experiments presented in the current study - and using preliminary results -, have also aimed the better understanding of virus prevalence and molecular analysis. 125 *Apodemus* mice persihed in traps served as a basis for our research topic. In the preliminary experiments, viral nucleic acid was extracted from the rodents' lung tissues. rRT-PCR (real time PCR) with primers specific for the nucleocapsid gene was used for proving the infection, amplifying a 433 bp long part of the S-segment. Out of the 125 samples, viral nucleic acid could be detected in ten cases (8%), seven originated from Af (7/58, 12%) while three from Aa (3/67, 4.5%).

Later, out of the ten positive samples, the ORF region of the complete S-segment could be amplified in eight cases (between nucleotides 36-1325, hence 1290 bp), encoding the nucleocapsid protein of 429 amino acids. In the phylogenetical analysis, we compared detected sequences to all available complete S-segment sequences of Aa and Af in the database of NCBI GeneBank.

Phylogenetical analyses showed that there are at least two circulating DOBV types in Central Europe, with partly different genome. New viral strains detected in Aa and Af form a monophyletic group with DOBV-Aa and DOBV-Af, respectively (Figure).

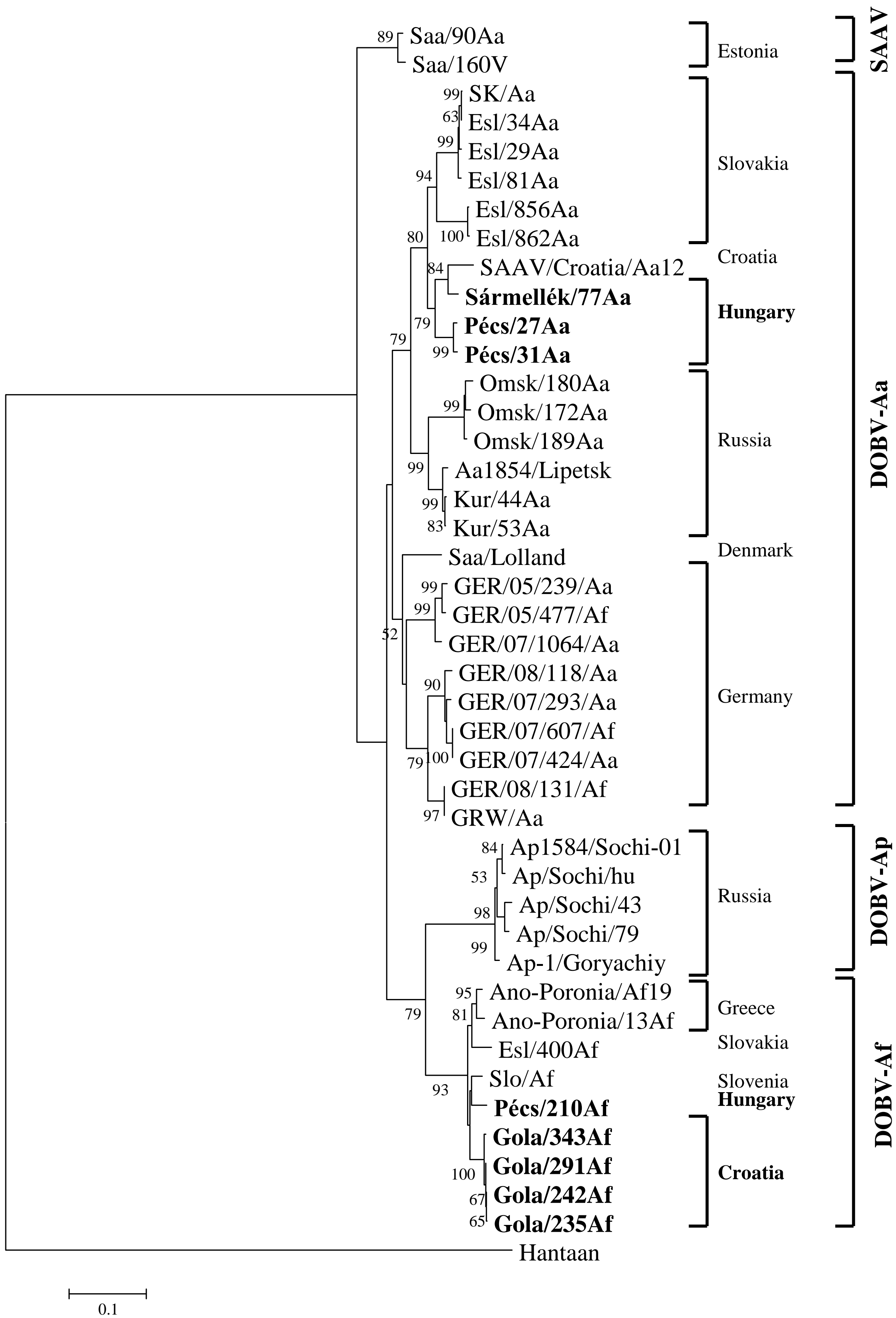

Figure. Phylogenetical analysis of DOBV S-segment (1290 bp) detected in rodents, using Maximum Likelihood method.

Viral strains we identified from Aa have a significant phylogenetic distance from SAAV, i.e. our results confirm suggestions regarding the debate of DOBV taxonomy. Plyusnin et al. were the first to detect SAAV in 1997 from *A. agrarius* rodents in the Estonian island of Saaremaa. Later, hantavirus pathogens were detected in several European countries, bearing significant difference from SAAV. Some scientists suggest that all viruses identified in Aa mice should be considered as SAAV, regardless of how far they are from the Estonian SAAV in phylogenetic sense. To resolve the issue, Klempa et al. in a 2012 publication proposed a way to distinguish genotypes within the DOBV species. According to them, among the Dobrava-Belgrade species, genotypes such as Dobrava, Saaremaa, Kurkino and Sochi should be discerned, depending on the geographical area of the first identification/isolation.

In several publications from the recent years, authors have drawn faulty conclusions regarding the relationships of viral strains, due to using an inadequate length of hantavirus sequences for phylogenetical analyses. Our results elucidate that in order to assess hypotheses in virus taxonomy, it is essential to use an adequately long, variable genome segment.

Recombinant protein expression

We have created a recombinant DOBV nucleocapsid protein, lacking the first 49 amino acids of the N-terminal (rNP50; amino acids 50-429). The protein expression system was optimized for *Escherichia coli*. The activity and antigen reactivity of the viral antigen was confirmed by Western blot assay. Specific monoclonal anti-HIS antibody sepcific for the 6x HIS tag at the N-terminal and polyclonal anti-DOBV human IgG was used in Western blot. We detected a signal with adequate intesity in both cases, indicating the active immunological state of the expressed protein.

During hantavirus infections, humoral immunresponse is stimulated to the largest degree by the viral nucleocapsid protein. Antibodies against the linear epitopes located at the N-terminal of the NP are not type-specific, resulting in a great cross-reactivity among members of the Hantavirus genus in various serological tests. Most variable regions are located between the amino acids 50-80 and 230-310. However, recombinant hantavirus nucleoproteins lacking the linear epitopes of the N-terminal, are perfectly suitable to distinguish different serotypes.

To be able to reliably use our ELSIA assay to test rodent and human serum samples, specificity and sensitivity had to be determined, compared to commercially available ELSIA tests, specific for DOBV and HTNV. A total of 50 human samples were used for the

comparison. Following the manufacturer's instructions, we got 8 positive and 42 negative samples for DOBV. By testing the same sample set with the rNP50-based in-house ELISA, we got 7 DOBV-positive findings. Out of the 42 negative samples, our test had resulted one false-positive. Based on these findings, specificity and sensitivity could be determined as 97.6% and 88.8%, respectively, in comparsion with the Reagena test from the market.

In summary, we have successfully utilized a truncated viral nucleocapsid protein for testing both human and animal samples.

Determining the prevalence of DOBV

The 125 *Apodemus* mice previously tested by rRT-PCR were also tested with our ELISA assay. Out of the 125 specimens, 21 animals (10 Aa and 11 Af), i.e. 17% were positive with at least one testing method. Among these, five animals (4%) were positive with both rRT-PCR and ELISA, also five (4%) were only positive for PCR and 11 rodents (8.8%) were ELISA-positive only. The phenomenon can be explained by three-stage infection progression, the characteristic for rodents. In the first stage, a few days post-infection, virus particles are rapidly replicating in lung tissues. This way, it is suggested that at the first stage, the pathogen is more likely to be detected by molecular methods. Immune response is starting later, thus antibodies against the virus are more difficult to identify. In the next stage, 10-14 days post-infection, the immune response is increasing, while virus titer is decreasing in the lungs. Hence, in the second stage, both molecular and serolgical methods can reliably detect viral infection. In the third stage, antiviral immun response expands to an extent that virus titer is nearly completely diminishing. According to the latter, mainly serological methods detect an infection.

Due to the discrepancies between the results of rRT-PCR and serology, we conclude that it is important to utilize both methods to gain reliable findings to estimate hantavirus prevalence in rodents. In earlier studies, usually only serologically positive samples were further investigated also by molecular detection methods. Hence, we suggest that hantavirus prevalence in these studies is underestimated. Plyusnina et al. (2009) investigated rodents captured in Hungary to estimate DOBV, SAAV and PUUV prevalence in the country. 10.5% of the total 362 *Apodemus* spp. tested (38/362) were positive by serological methods, with only additional two positives by RT-PCR. The rate of PCR-positive sampels was 0.5%, which value is 16 times greater, 8% according to our results. Research teams in the neighboring countries applied a similar way of testing. Plyusnina et al. (2011) carried out the above mentioned investigations also with samples from Croatia. They tested a total of 332 mice

from the *Apodemus* genus with Western blot assay, finding 20 positives (2 *A. agraius*, 6 *A. flavicollis* and 12 *A. sylvaticus,* 6.3% in total). When regarding only Aa and Af infections, they got 5% positivity (8/159). Lung tissues of the 20 positive animals were forwarded to molecular testing, confirming the two Aa infections, but only detecting viral RNA in 4 cases out of the 6 Af specimens. Avsinc-Zupanc et al. have surveyed DOBV prevalence and genetic variability back in 2000 (at the location of the first DOBV isolates), testing 260 *Apodemus* mice (231 Af, 21 Aa and 8 As). 20.4% of animals was seropositive with IFA and ELISA tests (53/260), out of which 49 were Af and 4 were Aa. Among these, 27 animals were positive for RT-PCR and an additional 23 rodents by nested PCR.

Outlook and further applications for the newly developed ELISA

Our ELSIA method based on a recombinant viral antigen provided us an opportunity not only to virologically investiagte rodent species, but also to carry out human/clinical sample testing with diagnostic purposes. We have established a close cooperation with several institutes of the Clinical Center of the University of Pécs, enabling us to test samples of patients hospitalized with susceptible hantavirus infection. We have proven several interesting infection cases during these experiments. We have confirmed previous serological findings also by molecular methods, which unequivocally proved hantavirus infection. We were able to first detect a DOBV infection mimicking acute appendicitis.

Our novel ELISA assay openend up the possibility of further serolgical-based investigations regarding transmission-dynamism among rodents. These studies greatly contributed to better understand virus spreading.

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