UNIVERSITY OF PÉCS

Biological and Sportbiological Doctoral School

Dynamics of temporal infection of human pathogenic hantaviruses in Hungary and determination of organ tropism in rodents

PhD Thesis

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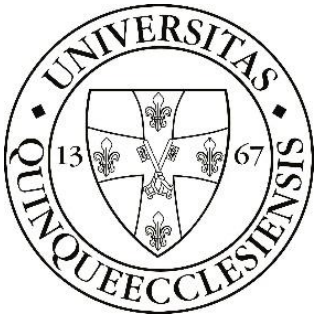

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1. INTRODUCTION

Nowadays, we pay special attention to zoonoses. Zoonosis is any infection that spreads from animals to humans, the pathogens of which can be of bacterial, viral, or parasitic origin. According to the World Health Organization (WHO), 75% of emerging infectious diseases in the last two decades are of zoonotic origin. Hantaviruses are pathogens of public health importance both in Hungary and worldwide. In view of the fact that although the number of cases per year is not very high, they can cause very serious illnesses.

Hantaviruses are classified within the subfamily *Mammantavirinae* in the *Bunyavirales* order. Virions are spherical, enveloped, with a diameter of 80-120 nm. They have three negative single-stranded RNA segments as a genome that is approximately 12 kilobases in length. The small (S) segment encodes the nucleocapsid protein, the medium (M) segment, the Gn and Gc glycoproteins, and the large (L) segment encodes the RNA-dependent RNA polymerase (RdRp). At room temperature, the virus from the host is able to maintain its infectivity for 12-15 days, which is of paramount importance for the spread of the virus. Among hantaviruses, we distinguish between New World and Old World viruses. Hantaviruses in Europe, Asia and Africa are are known as “Old World”. These viruses cause HFRS (hemorrhagic fever with renal syndrome), a hemorrhagic fever associated with acute renal failure, and the NE (nephropathia epidemica) which is a milder form of HFRS. HFRS is caused by *Dobrava-Belgrade virus* (DOBV) in Europe, *Hantaan* (HTNV) and *Seoul virus* (SEOV) in Asia. Whereas *Puumala virus* (PUUV) is responsible of NE. In parallel, the New World group includes hantaviruses in North and South America, which cause HCPS (hantavirus cardiopulmonary syndrome), a hemorrhagic fever with severe pulmonary hemorrhage and pulmonary oedema, with a mortality rate of 20-50%. The most well-known HCPS-causing viruses are *Sin Nombre* (SNV) and *Andes virus* (ANDV). The virus spreads through the body secretions (saliva, feces, urine) of infected animals. The infection can occur either directly between humans and animals, e.g., by biting or indirectly through the respiratory tract, during which dust contaminated with the body secretions of the infected animal enters the lungs. The disease is not expected to spread from person to person.

The hosts of the virus within the order Rodentia are mice (*Muridae*) and voles (*Cricetidae*), *Eulipotyphla* (formerly known as insectivores) shrews and moles (*Soricidae*, *Talpidae*) in addition to the chiropteran order (bats). Hantaviruses transmitted by rodents have clinical relevance. More than 9,000 cases of HFRS are diagnosed in Europe each year and this number is growing. In northern Europe, the cycle of HFRS epidemics is 3-4 years,

which is the same as the cyclicality of rodent populations. Although the number of cases varies from country to country. Hantaviruses that cause HFRS are present across Europe. Among the human pathogenic viruses, DOBV is known to be present in Hungary, which is spread by the yellow-necked wood mouse (*Apodemus flavicollis*), the black-striped field mouse (*Apodemus agrarius*) and the wood mouse (*Apodemus sylvaticus*). The host of PUUV is the bank vole (*Myodes glareolus*). The human pathogenicity of *Tula virus* (TULV) has long been questionable, but more and more studies confirm the fact of human pathogenicity. TULV is carried by common voles (*Microtus arvalis*) and field voles (*Microtus agrestis*). In rodents, after the initial acute phase or viremia, the virus multiplies in the target organs (lungs, kidneys, liver, spleen, salivary glands) and then replication decreases but remains constant, although anti-viral antibodies are already present in large amounts. IgG antibodies against the virus can persist for the rest of their lives. According to a Finnish study, rodents are able to excrete the virus in their urine, feces and saliva for life-long. The virus does not spread vertically among rodents, but the presence of maternal antibodies against hantavirus greatly influences the infection, as the animals are thus protected against infection for the first 6–12 weeks of their lives. The extent of hantavirus infection in the areas is influenced by a combination of several factors, the most important of which are rodent abundance, age, sex, and environmental factors such as climate, temperature, and food intake. According to one study, in addition to the aforementioned factors, hantavirus infection negatively affects the winter survival of animals.

2. AIMS OF THE STUDY, QUESTIONS

One of the main aims of the dissertation is to investigate the dynamics of the temporal infection of two human pathogenic hantaviruses detected in rodent species in the Kőszegi-forrás Forest Reserve, based on the following research tasks and questions:

- multi-annual monitoring of rodent species by live trapping.
- once-monthly blood draws from captured individuals from the retroorbital vein.
- Perform ELISA tests to detect DOBV and PUUV antibodies.
- To determine the correlations and differences between the characteristics of the host population and the degree of viral infection carried out by determining the seroprevalence of DOBV and PUUV.

Questions:

- Does the change in seroprevalence depend on the individual density of the host?
- Does the change in seroprevalence over time show synchronization in the comparison of hantavirus species?
- Does seasonality affect the time dynamism of the infection?
- Is there a gender difference in infection?
- Does the infection affect the chances of the animals surviving the winter?

The other main goal of the dissertation is to study the tissue tropism of hantaviruses in Hungary, by applying the following protocol:

- Necropsy of dead animals, especially for the removal of urine.
- Detection and determination of hantaviruses by molecular biological methods in various organs and urine.
- Use of a hantavirus-specific serological test.

Based on this protocol, we examined the hypothesis of ‘lifelong virus shedding’ in rodent hosts. On the other hand, this study also focused on the methodological problem of which organ in the host animal is the most effective in detecting viruses, which contributes to the optimization of autopsies and the collection of organ preparations.

3. MATERIALS AND METHODS

Tested rodents

In our research on hantavirus, we primarily examined rodent species that are known carriers of the virus. The trapping and survey of small mammals was carried out by the staff of the Small Mammal Research Group of the Department of Ecology, Institute of Biology, University of Pécs.

Sampling areas, trapping, blood sampling

For the study of infection dynamics, ecologists carried out traps in Kőszegi- forrás Forest Reserve in the Mecsek Mountains for four years. In addition, we processed samples from rodents from Pécs, Beremend, Vajszló and Kis-Balaton. The live trap boxes were placed 5 m apart using a 6 × 6 trap net. Capture-mark-recapture (CMR) method was used, so that the infection of the animals could be monitored from month to month. During trapping, blood was taken from each captured animal once a month from the animals 'retroorbital vein with a glass capillary. Serum and any animals that died in the traps were stored at -20 ° C.

Autopsy, viral nucleic acid isolation, PCR, sequencing

Animals may also die in the trap when using the live trap method. The organs of the animals that died in the trap could be used for further experiments, such as dissections in rodents, during which the lungs, liver, spleen, and kidneys were removed from the abdominal cavity. If possible, urine in the bladder was aspirated with an insulin syringe and placed in microcentrifuge tubes. Viral nucleic acid was extracted from organs and urine using a Geneaid viral nucleic acid extraction kit according to the manufacturer's instructions. PCR was performed using hantavirus L segment specific primers and amplicons were visualized by agarose gel electrophoresis. The sequencing reaction was performed using a BigDye™ Terminator v1.1 Cycle Sequencing Kit with an ABI Prism 310 automated DNA sequencer.

Serological tests

Detection of immunoglobulin G (IgG) antibodies against DOBV and PUUV in rodent blood samples was performed using an ELISA (enzyme linked immunosorbent assay)

system developed by our research team, and the results were confirmed by Western blotting (WB).

Statistical surveys

Differences, correlations, annual and seasonal analyzes of the number of individuals per genus (*Apodemus, Myodes*) tested and infected were performed using STATISTICA 12 software using Pearson's Chi-square, Wald Wolfowitz and Kruskall-Wallis tests. A boxplot diagram was used to represent seroprevalence values. Significance was determined to be $P \leq 0.05$ for each test performed.

5. NEW SCIENTIFIC RESULTS

In my dissertation I studied the hosts of viruses causing hantavirus infection in Hungary from several points of view. A four-year dynamic study of infection was performed in the area of the Kőszegi- forrás Forest Reserve, and animals killed in traps during small mammal monitoring were examined using molecular biological and serological methods to detect hantavirus infection.

Infection dynamics test results

Based on our preliminary hantavirus research results, from the spring of 2011 to the fall of 2014, ecologists continued to monitor rodents. 3598 blood samples were examined from 2491 individuals of the four species tested for viral infection. The species distribution of the individuals in our study was as follows: 1749 (70.2%) *Apodemus flavicollis*, 517 (20.8%) *Myodes glareolus*, 180 (7.2%) *Apodemus sylvaticus* and 45 (1.8%) *Apodemus agrarius*. The sex distribution was nearly equal for all four species. The difference in the number of individuals of DOBV host *Apodemus* species showed a significant difference, similar to the previous studies ($\chi2 = 1276,185$; df = 1; P <0.001). Since all three species are hosts of the *Dobrava virus*, they were grouped at the genus level, so further analyzes were performed between *Apodemus* species carrying DOBV and *Myodes glareolus* carrying PUUV.

Of the individuals studied over four years, *Apodemus* were significantly more present than *Myodes* ($\chi2 = 852,207$; df = 1; P <0.001). Of all the individuals tested (2491), 254 showed seropositivity for hantavirus. A further 31 animals showed seropositivity, but these were clearly individuals with maternal antibodies, so these animals were omitted from further analyzes. There was a significant difference ($\chi2 = 127,559$; df = 1; P <0.001) between the number of seropositive mice (217; 85%) and the number of voles (37; 15%), but the mean hantavirus seroprevalence over four years was almost the same ($\chi2 = 0.001$; df = 1; P = 0.968). We observed a large difference between the number of animals collected in different years. This can be traced back to the dynamism of the rodent population. In the first two years we were able to study an average of 1000 animals, while in the third year the number of individuals decreased to one third of the previous years due to the decrease in population abundance, then in the fourth year there was a significant increase ($\chi2 = 43.811$; df = 1; P <0.001). In the first year of monitoring, 17.25% (114/661) of *Apodemus* mice showed seropositivity, compared with only 3.9% (3/77) of *Myodes glareolus*. In 2012, the

number of examined individuals was significantly higher ($\chi 2 = 110{,}556$; df = 1; $P < 0.001$), but the seroprevalence of DOBV decreased to 10.2% (88/864), while in the case of PUUV a slight increase was observed (5.3% 18/337). The collapse of the populations brought an interesting change, as in 2013 the infection of *Apodemus* carrying DOBV decreased to 5.3%, while this value increased further in *Myodes* cases (8%). Despite the increase in the *Apodemus* population in 2014, the seropositivity of DOBV decreased further (3.6%), in contrast, although there was no significant increase in the abundance of the *Myodes* population, PUUV infection doubled compared to the previous year (18.5%). Based on the annual analysis of the data, the hantavirus infection in the area decreased but did not fall below 5%, which may be due to the fact that the seroprevalence of the two rodent groups was reversed during the four years, so the infection decreased but did not disappear. In a seasonal analysis, we observed that there was no difference in the infection of different seasons within a given year, while in the analysis of the same seasons of different years, we observed a decrease in seroprevalence of DOBV. In the case of PUUVs, the data from the voles are not suitable for drawing clear conclusions due to the very different numbers of traps per season. Using the CMR method, seroconversion was observed in 56 cases and seroreversion was observed in 3 cases. Although we can observe a difference in the level of infection between the sexes in 2011, in 2012 this value shows a significant difference, and by 2014 it will level off in the case of both hosts. There was no significant difference in winter survival between seropositive and seronegative animals.

Tissue tropism test results

Between 2011 and 2015, the Small Mammal Research Group carried out small mammal monitoring in five areas of South Transdanubia, after which 665 dead animals were necropsied. Urine was found in the bladders of 163 animals, so we further examined our 163 animals. The animals studied were individuals of 7 different species: 22 (13.5%) *Apodemus agrarius*, 64 (39.2%) *Apodemus flavicollis*, 6 (3.7%) *Apodemus sylvaticus*, 53 (32.5%) *Myodes glareolus*, 6 (3.7%) *Microtus agrestis* (field vole), 11 (6.7%) *Microtus arvalis* (common vole) and 1 (0.6%) *Arvicola amphibious* (European water vole). Of the 163 rodents, 25 (15.3%) were able to detect the presence of hantavirus and / or IgG antibody against the virus in the animal.

Using nested-PCR (nRT-PCR), we were able to detect the presence of the virus in necropsy (lung, liver, kidney) or urine in 20 animals.

Of the organs examined, viral nucleic acid could be detected in all kidneys (20/20; 100%) and in only 11 cases (11/20; 55%) in the lungs. The virus is probably present in the urine only for a short time, as only 3 of the 20 urine tested positive for hantavirus (3/20; 15%). Hantavirus was detected in only 15% of the urine, so although little data are available, we cannot clearly confirm the hypothesis of urinary life-long virus shedding.

During the serological tests, we only had a test for the detection of antibodies against *Dobrava* and *Puumala virus*, so we did not perform a serological test with *Microtus* species, which are the hosts of the *Tula virus*, for this reason the total number of individuals in the further serological analysis was 21.

During the serological examination by Western blot (WB) technique, we were able to detect IgG antibody against the virus in 18 of the 21 hantavirus-positive samples, and the virus was detectable in only 3 animals. In five animals, only antibodies could be detected, so no viral nucleic acid, so these individuals were only seropositive. Using Sanger sequencing, the infectious hantavirus species in each animal were determined. *Dobrava-Belgrade* was detected in *Apodemus* mice without exception, *Puumala virus* in *Myodes glareolus*, and *Tula virus* in *Microtus* voles with 89%, 95%, and 100% homology.

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7. PUBLICATIONS

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