Detailed results

Basically, there were two main questions in the grant proposal. First, the study of the transcription and function the genes and gene products of fish viruses. Second is the study of unknown diseases (with viral origin) occurring in fish hatcheries, the isolation, the identification and the taxonomical classification of the causative agents.

1.1. Structural study and enzyme activity assays for proteins of WSAdV-1:

The WSAdV-1 genome contains two ORFs, whose putative protein products show homology to sulfotransferases (so far, such genes were not found in any viral genomes) and encode four putative fiber genes. Usually, AdV capsid contains only one fiber per vertex. From the vertex of aviadenoviruses two fibers protrude. The other fowl AdVs genomes contain only one, but their gene products are duplicated on the vertexes. Some human and non-primates monkey AdV genomes comprise two fiber genes, but only one fiber protrudes from the vertexes. The isolation of these genes was carried out by PCR and they were cloned into Pet28 expression vectors for later enzyme activity assays and 3D structural studies (X-ray chrystallography). Altogether 14 different constructions were tested, all of them expressed the proteins in a good quantity, unfortunately all of them proved to be indissoluble. These days, I am trying to clone a SUMO (small ubiquitin-like modifier) gene into the constructs, which could help to turn the proteins soluble. If this approach fails, I am going to give a try to express them in baculovirus expression system.

1.2. Transcriptome and gene expression analysis:

We have very limited knowledge about the transcription and gene expression profile of fish herpesviruses. So far, only one member of the family *Alloherpesviridae* was studied; the AngHV-1 from the genus *Cyprinivirus*. We started to carry out the transcriptome analysis and gene expression study of the Ictalurid herpesvirus 2 from the genus *Ictalurivirus* in order to increase our acquaintance on this field. The IcHV-2 was propagated on EPC cell line, and its complete genome was sequenced by 2nd generation method and analysed. We

designed qPCR for each gene and did the time dependent RNA isolation. The qPCRs are in the pipeline now.

1.3. DNA vaccine against SbSHV:

Development of some DNA-vaccine candidates against Siberian sturgeon herpesvirus (SbSHV) has been carried out within a Russian collaboration. Some of the genes coding capsid, membrane and tegument proteins (ORF39, ORF46 and ORF59) were amplified and sequenced. Subsequently DNA-vaccines were designed and produced using the amplified genes and pcDNA3.1 plasmid. The vaccine candidates were tested in Russia. Group of 25 sturgeons (100 g mean body weight) were intraperitoneally injected by the DNA vaccines (1mg/kg fish). One of the candidates (ORF46) produced promising results. While the survival ratio was only 4% in the control group 70 days post infection, that of in the group treated by ORF46 DNA-vaccine was 20%. Further studies/optimisation with this candidate is planned.

2.1. Atlantic salmon papillomatosis:

Papillomatosis of Atlantic salmon (*Salmo salar*) has been reported for decades in Russia, Scandinavia and Scotland. The disease is typically benign although heavy losses have occasionally been reported. A herpesviral etiology has been suggested based on ultrastructural evidence; however, the virus has not been isolated or genetically characterized. A Russian collaborator sent us samples from diseased salmons for molecular study. We were able to amplify couple of genes partially and provide the first viral sequences detected in the papillomas from diseased Atlantic salmon. Phylogenetic analyses, based on the partial sequences of the herpesviral polymerase and terminase genes, supported the virus as a novel member of the genus *Salmonivirus* within the family *Alloherpesviridae*. The sequences of the Atlantic salmon papillomatosis virus differ markedly from those of the three known salmoniviruses, therefore the authors proposed the *Salmonid herpesvirus 4* species designation to be considered for approval by the International Committee on Taxonomy of Viruses. (Doszpoly A, Karaseva TA, Waltzek TB, Kalabekov IM, Shchelkunov IS (2013) Atlantic salmon papillomatosis in Russia and molecular characterization of the associated herpesvirus. Dis Aquat Org 107(2):121-127).

2.2. Cyprinid herpesvirus 4:

In the early summer of 2014, mass mortality of Sichel (*Pelecus cultratus*) was observed in Lake Balaton, Hungary. Histological examination revealed degenerative changes within the tubular epithelium, mainly in the distal tubules and collecting ducts in the kidneys and multifocal vacuolisation in the brain stem and cerebellum. The routine molecular investigations showed the presence of the DNA of an unknown alloherpesvirus in some specimens. Subsequently, three genes were amplified and sequenced partially from the putative herpesviral genome (DNA polymerase, terminase, and helicase). Phylogenetic tree reconstruction, based on the concatenated sequence of these three conserved genes, implied that the virus undoubtedly belongs to the genus *Cyprinivirus* within the family *Alloherpesviridae*. The sequences of the Sichel herpesvirus differ markedly from those of the three known cypriniviruses (CyHV-1, CyHV-2 and CyHV-3); putatively representing the fourth virus species in the genus. (Doszpoly A, Papp M, Deákné PP, Glávits R, Ursu K, Dán Á (2015) Molecular detection of a putatively novel cyprinid herpesvirus in Sichel (*Pelecus cultratus*) during a mass mortality event in Hungary. Arch Virol 160: 1279-1283)

2.3. North-American sturgeon herpesviruses:

The genomic comparison of different acipenserid herpesvirus 2 (AciHV-2) strains was carried out. The strains were isolated from white sturgeon (*Acipenser transmontanus*), lake sturgeon (*A. fulvescens*), and shortnose sturgeon (*A. brevirostrum*) in the USA. The genome of the white sturgeon AciHV-2 Californian strain was completely sequenced (providing the first full genome from sturgeon herpesviruses), while that of the other two strains only partially, an 8 kbp long fragment between the DNA polymerase and the terminase genes. For the comparative analysis AciHV-2 strains isolated from Siberian sturgeon (*A. baeri*) originating from Russia and another white sturgeon AciHV-2 strain (SRWSHV) were also involved. Generally speaking, herpesviruses and alloherpesviruses are host specific viruses, which have co-evolved with their hosts. It seems that the AciHV-2 has a broader host range and it is able to multiply and cause severe disease with high mortality at least in five sturgeon (*Acipenserid*) species. (manuscript in preparation)

2.4. Siberian sturgeon herpesvirus:

Siberian sturgeon herpesvirus (SbSHV) was isolated in Russia for the first time in 2006. Nine SbSHV isolates were recovered from different fish hatcheries producing the same CPE in cell cultures, the same clinical signs and mortality kinetics in virus-infected fish, the same virus neutralization pattern, and shared identical nucleotide sequences. In 2011 a new isolate was recovered from juvenile sturgeon, which caused completely different CPE. That isolate was not readily neutralized by Siberian sturgeon hyperimmune antisera and its DNA was not recognized by the routine PCR developed for SbSHV detection. Molecular study of the novel isolate revealed that it was more closely related to North-American Acipenserid herpesvirus 2 (AciHV-2) isolates from white sturgeon, while the genome sequences of the former SbSHV isolates showed high similarity to the AciHV-2 isolated from shortnose sturgeon. While clinical signs and mortality caused by the novel isolate in infected Siberian sturgeon were similar to those of the formerly described SbSHV isolates, the incubation period and mean time to death produced by the novel isolate were twice as long. The differences between the former isolates and the recent one suggest that a novel SbSHV strain emerged in Europe and the molecular findings imply its North-American origin. (Doszpoly A, Kalabekov IM, Breyta R, Shchelkunov IS (2017) Isolation and characterization of an atypical Siberian sturgeon herpesvirus strain in Russia: novel North American Acipenserid herpesvirus 2 strain in Europe? J Fish Dis doi:10.1111/jfd.12611)

2.5. Eel circovirus detection:

An adult European eel (*Anguilla anguilla*) originating from Lake Balaton, showing typical signs of the so-called cauliflower disease, was subjected to pathological and molecular virological examinations. Samples, taken from the internal organs and the polypoid proliferative tissue from the mouth, were examined by PCR for the detection of several viruses. Positive results were obtained with a nested PCR targeting the rep gene of circoviruses. Analysis of the partial rep sequence indicated the presence of a putative novel circovirus, but attempts to isolate it remained unsuccessful. The missing part of the genome was acquired by an inverse nested PCR with two specific primer pairs, designed from the newly determined rep sequence, then genome walking was applied. The circular full genome was found to consist of 1378 nt. Two

oppositely oriented ORFs were present. One of them could be identified as a circoviral rep gene unambiguously. However, the predicted product of the other ORF, though it is a clear positional counterpart of the cap genes, showed no obvious homology to any known circoviral capsid proteins. A stem-loop-like element in the intergenic region between the 5' ends of the ORFs was also found. Phylogenetic calculations indicated that the novel virus belongs to the *Circovirus* genus of the *Circoviridae* family. The relative amount of the viral DNA in the organ samples was estimated by quantitative real-time PCR. The results suggested that the examined fish was caught in an active viraemic status, albeit the role of this circovirus in the etiology of the cauliflower diseases could not be ascertained. (Doszpoly A, Tarján ZL, Glávits R, Müller T, Benkő M (2014) Full genome sequence of a novel circo-like virus detected in an adult European eel *Anguilla anguilla* showing signs of cauliflower disease. Dis Aquat Org 109: 107-115)

2.6. Further studies on eel circovirus:

The prevalence and distribution of piscine circoviruses (CVs) were tested in a routine virus monitoring program in Lake Balaton, Hungary. A high prevalence of European eel CV (EeCV) was found in the apparently healthy eel population (35.5%). The copy number of the viral DNA in different organs was determined by quantitative real-time PCR. The results suggested that some eel specimens were in active viraemic status despite their asymptomatic condition. Furthermore, a novel, previously undescribed CV was also detected in eel and sichel samples. Full genome characterisation confirmed that the virus represents a novel EeCV species (EeCV-2). The genome contains an integrated eel chromosome-derived fragment, suggesting that the original host of the virus was the eel and it probably emerged subsequently in the sichel by host switching. In some samples, an additional, 1111-nt-long circular ssDNA was also observed involving a CV-like stem-loop structure and an ORF showing similarity to CV capsid protein genes, without any sign of a replication initiator protein sequence. (manuscript has been submitted to Acta Veterinaria Hungarica)

2.7. Bullhead ranaviruses

Ranaviruses are emerging pathogens associated with high mortality diseases in fish, amphibians and reptiles. Here we describe the whole genome

sequence of two ranavirus isolates from brown bullhead (*Ameiurus nebulosus*) specimens collected in 2012 at two different locations in Hungary during independent mass mortality events. The two Hungarian isolates were highly similar to each other at the genome sequence level (99.9% nucleotide identity) and to a European sheatfish (*Silurus glanis*) origin ranavirus (ESV, 99.7%–99.9% nucleotide identity). The coding potential of the genomes of both Hungarian isolates, with 136 putative proteins,were shared with that of the ESV. The core genes commonly used in phylogenetic analysis of ranaviruses were not useful to differentiate the two brown bullhead ESV strains. However genome-wide distribution of point mutations and structural variations observed mainly in the non-coding regions of the genome suggested that the ranavirus disease outbreaks in Hungary were caused by different virus strains. At this moment, due to limited whole genome sequence data of ESV it is unclear whether these genomic changes are useful in molecular epidemiological monitoring of ranavirus disease outbreaks. Therefore, complete genome sequencing of further isolates will be needed to identify adequate genetic markers, if any, and demonstrate their utility in disease control and prevention. (Feher E, Doszpoly A, Horvath B, Marton S, Forro B, Farkas SL, Banyai K, Juhasz T (2016) Whole genome sequencing and phylogenetic characterization of brown bullhead (*Ameiurus nebulosus*) origin ranavirus strains from independent disease outbreaks. Infect Genet Evol 45: 402-407)

2.8. EVE study:

Endogenous viral elements (EVEs) are entire or fragmented viral genomes that have been integrated into the genome of their hosts and are therefore vertically inherited in a stable manner. Recent studies have uncovered plenty of viral sequences that are integrated in the genomes of eukaryotes. It seems that not only retroviruses but almost all types of viruses can become endogenous. However, there are very few reports on herpesviruses which integrated into the host genome (some human and primate examples). Using broad spectrum PCRs for detection of fish herpesviruses give us many times false positive results. Sequencing and analyzing these false positive results revealed that herpesviral EVEs are very common among fish species. I studied mostly salmonid and sturgeon species. 15 species were examined from the two fish families; all of them contained herpesviral sequences. Later I carried out in

silico studies, which showed that other fish taxa also contain herpesviral EVEs, moreover some fish species contain almost complete herpesviral genomes. With RT-PCR I was able to detect mRNAs of the above mentioned EVEs from permanent fish cell lines. I` d like to continue the study of these EVEs in order to get insights into the origin, evolutionary dynamics and structural evolution of fish herpesviruses, which could contribute to the novel field of palaeovirology. (manuscript in preparation)

2.9. Recent findings

After a mortality event among Siberian sturgeon fingerlings in a Hungarian farm, we isolated a virus on EPC cell line. The virus was propagated and subsequently nucleic acid extraction was carried out, then the sample was submitted for 2nd generation sequencing. The preliminary data suggest that the isolated virus is closely related to the Tasmanian aquabirnavirus. Full genome assembly is needed to establish the taxonomic status of the novel virus (in the pipeline).

Cyprinid herpesvirus-1 (carp pox) is a well know herpesvirus isolated from carp and koi carp. Recently we were able to detect a virus (showing 99% nucleotide similarity to CyHV-1 in the polymerase gene) in barbel (*Barbus barbus*) caught in river Danube. This is the first report of the CyHV-1 occurrence in other fish species than carp. The amplification and sequencing of other genes are in the pipeline.