PROJECT CLOSING REPORT

About the OTKA research project entitled:

„Reptilian and avian reoviruses: diversity, evolution and classification”

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In our OTKA research project, entitled „Reptilian and avian reoviruses: diversity, evolution and classification" we aimed to study reptilian and avian orthoreoviruses of Sauropsids' (birds and reptiles) -a group of the Amniote clade- in order to discover the diversity of the genus *Orthoreovirus*, to understand their evolution and to describe their classification more precisely. Despite the fact that avian and reptilian reoviruses cause significant economic losses in poultry farms and reptile collections, and novel variants are threatening the conservation programs of endangered species, our knowledge about these viruses was scanty at the beginning of our study. Reoviruses have developed a number of molecular mechanisms which facilitates the quick change of their genetic material, host transmission events and modification of their phenotype. In our project, we aimed to explore these mechanisms. With the help of the newly accumulated sequence data, more efficient diagnostic systems and prevention strategies can be developed.

Reptilian orthoreoviruses

Despite the fact that reptilian orthoreoviruses are frequently detected from different samples, at the beginning of our study only partial sequence data was available in GenBank. Therefore, we aimed to determine and analyze the complete genome sequences of strains isolated from Hungarian samples and strains kindly provided by our German collaborators, respectively. First the genome sequence of strain 47/02, isolated from a green bush viper (*Atheris squamigera*), had been analyzed, and was later used as the first representative of the *Reptilian orthoreovirus* species in our analysis. The genomic organization of 47/02 was similar to that of other orthoreoviruses. Based on sequence analysis and phylogenetic calculations performed with each genomic segments close genetic relationship could be observed between 47/02, the Broome virus and the Baboon orthoreovirus, indicating that these viruses might have been originated from the same ancestor.

The complete genome sequence of a strain isolated from a terrestrial tortoise species (*Testudo graeca*) had been determined and analyzed. The genomic organization of CH1197/96 was similar to and corresponded with that of the bush viper reovirus. Although the common origin of the two reptilian strains could be concluded based on the phylogenetic calculations performed with all genomic segments, nucleotide and amino acid sequence similarity values were lower than expected. For classification of strains into species in the genus *Orthoreovirus* the International Commitee on Taxonomy of Viruses has determined nucleotide and amino acid sequence identity values. The genetic distance between 47/02 and CH1197/96 proved to be greater than expected between members of a certain species, which may question the accuracy of the currently used classification system.

During the 4 years of our project the complete genomic sequence of 8 reptilian orthoreovirus strains had been determined. Based on the phylogenetic calculations, the strains formed 3 main clades. The novel sequence data provided additional information about reptilian orthoreoviruses, which had previously been limited mainly to their biological properties. We confirmed the taxonomic position of reptilian orthoreoviruses and broadened our knowledge about the evolution of members of the genus *Orthoreovirus*.

Avian orthoreoviruses

At the beginning of our research project the complete genome sequence of 6 avian orthoreovirus strains was available in GenBank. The complete genomic sequence of more than 100 chicken, turkey, goose, Muscovy and Peking duck, crow, partridge and pheasant reovirus isolates had been determined by next generation sequencing to obtain more information about their genomic organization, evolutionary mechanisms, and phylogenetic and epidemiological relationships. The isolates originated from different countries, Hungary, France, Germany, Finland and the US, respectively. Dozens of viral strains were studied in collaboration with the CEVA-Phylaxia. In the course of our project we could observe a much greater genetic diversity than we previously assumed.

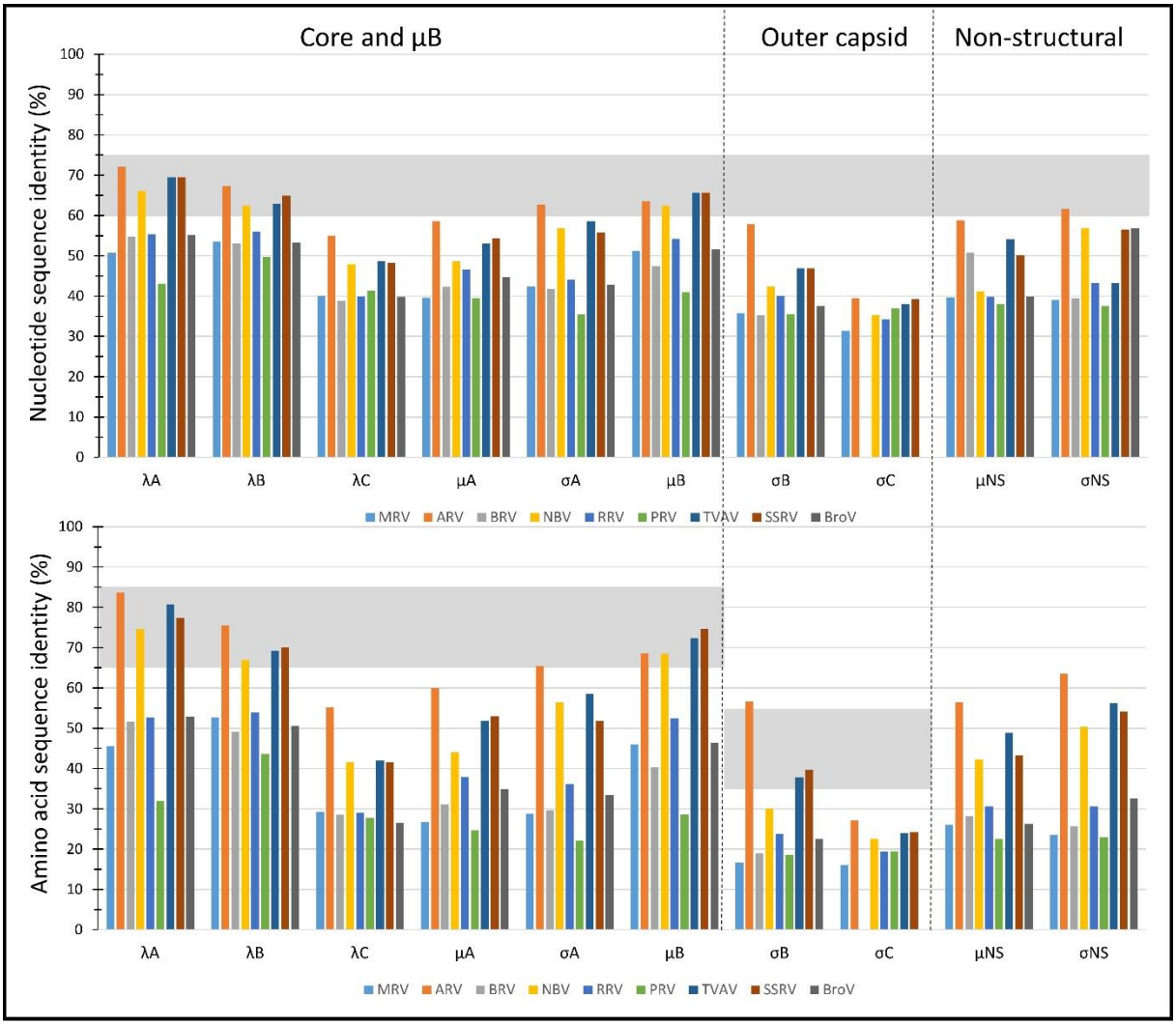
Waterfowl orthoreoviruses

A goose reovirus strain, D20/99, had been isolated in Hungary in 1999. We aimed to investigate the genetic relationship between the Hungarian goose strain and the Far East duck and goose strains. Results of the sequence and phylogenetic analyses indicated the different origin of certain genomic segments. With the exception for the genes encoding μA, μNS and σA, the other genes showed the highest degree of similarity with classical and novel waterfowl orthoreovirus strains detected in the Far East. At this time only partial sequence data was available from European strains that we could include in our analysis. The genomic composition of the goose reovirus strain referred to reassortment events that might have occurred in the past. We could not observe geographical separation of the strains on the phylogenetic trees.

Analysis of the complete genomic sequences of two French Muscovy duck reovirus strains (D1546 and D2044) had been performed to obtain information about classical and novel waterfowl orthoreoviruses originating from different continents, and the epidemiological relationships between European and Asian strains. Strain D1546 was isolated in 2010 from a 43-day-old Muscovy duckling originating from a flock showing the clinical signs characteristic to runting-stunting syndrome, as well as locomotor disorders and feather abnormalities. D2044 was isolated in 2012 from a 17-day-old Muscovy duckling with locomotor disorder and respiratory distress. The two strains showed the highest degree of similarity with each other and on the phylogenetic trees grouped together with other European and Asian waterfowl strains. Based on the phylogenetic analysis performed with all genomic segments, we could identify several potential reassortment events between classical and novel Chinese and European waterfowl strains and between waterfowl and chicken origin reovirus strains.

In addition to the French samples, complete genome sequencing of reovirus strains isolated from Pekin ducks in Germany was also performed. Based on the sequence analysis performed with all genomic segments and the results of the phylogenetic calculations, strain, D2533/4/1-10, proved to be a triple reassortant. According to the sequence similarity values, 6 segments of the D2533/4/1-10 strain (λA, λB, λC, μA, μNS and σNS) showed the highest degree of similarity with the classical waterfowl orthoreovirus strains, while 3 segments (μB, σB and σC) with novel waterfowl strains, respectively. In contrast, the σA segment showed nearly identical similarity values with both classical and novel types of waterfowl orthoreoviruses. In case of this gene separation of the classical and novel types could not be observed due to the high number of reassortment events in the past. Although σC showed the highest degree of sequence similarity with the novel type of waterfowl orthoreoviruses, on the phylogenetic tree the strain appeared on a separate branch, indicating that this segment might be originating from

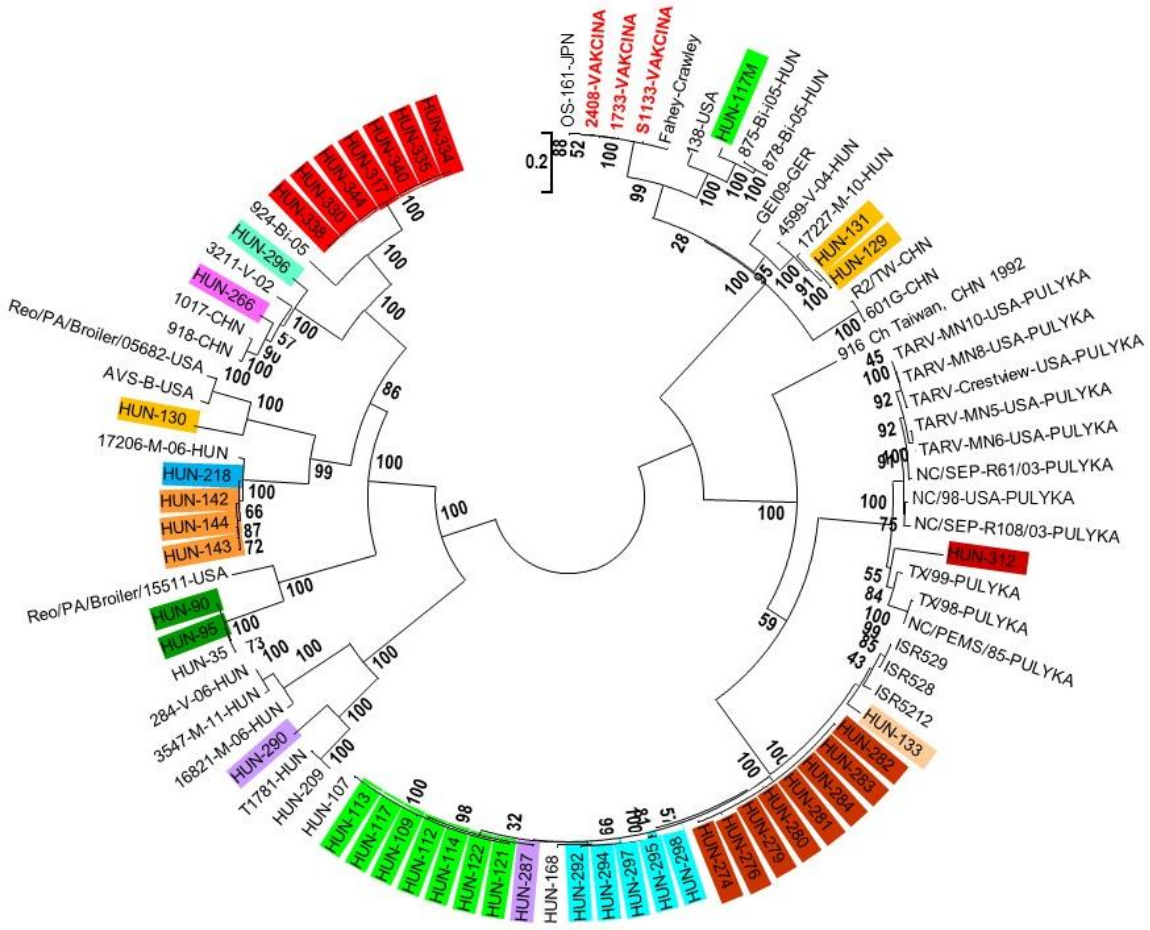
a yet unknown waterfowl orthoreovirus. The different origin of S1 was also supported by the tricistronic structure of the segment, which has only been observed in chicken and turkey origin reoviruses and in novel waterfowl orthoreoviruses. The other strain D2533/6/1-10 proved to be only distantly related to previously described avian orthoreoviruses.



Comparative diagram based on the percentile nucleotide and amino acid sequence identities of different genome segments between the strain 2533/6/1-10 and the representative strains of six *Orthoreovirus* species (*Mammalian orthoreovirus*, MRV: Mammalian orthoreovirus 1 strain Lang; *Avian orthoreovirus*, ARV: Avian orthoreovirus strain S1133; *Nelson Bay orthoreovirus*, NBV: Nelson Bay virus; *Reptilian orthoreovirus*, RRV: Bush viper reovirus strain 47/02; *Baboon orthoreovirus*: BRV: Baboon orthoreovirus), *Piscine orthoreovirus* (PRV): Piscine orthoreovirus strain Salmo/GP-2010/NOR and three unclassified orthoreovirus strains, Broome virus (BRV), Steller sea lion reovirus (SSRV), and Tvärminne avian virus (TVAV), respectively. The bars are ordered according to the virus list at the bottom. (A) The grey area indicates the species demarcation cut-off values (60–75%). (B) The grey areas indicate the species demarcation cut-off values for the more conserved core proteins plus the µB protein (65–85%), and for the outer capsid proteins (35–55%), respectively. No cut-off values have been defined for the non-structural genes indicating the lack of consensus concerning their role in virus taxonomy.

Chicken orthoreoviruses

In cooperation with the NÉBIH ÁDI in Debrecen, we had the opportunity to study 10 chicken reovirus isolates randomly selected from a strain collection. The near complete genome sequence of the strains had been determined and analyzed. Isolates were collected from different farms in Hungary between 2002 to 2011. The aim of the study was to assess the genetic diversity of strains circulating in the Hungarians farms, to discover their epidemiological relationships and to explore their evolutionary mechanisms. 8 strains showed the highest degree of similarity with the previously isolated and characterized Hungarian strain, T1781, while 2 strains showed close relationship with the US origin AVS-B strain. Based on the topology of the phylogenetic trees we could identify a number of potential homologous reassortment events between strains with different geographic origin. Unexceptionally analysis of the μB gene revealed a potential heterologous reassortment event in case of 3 Hungarian strains. Analyzing the genomic segments of the Hungarian isolates and chicken origin reovirus strains available in GenBank, we could identify 12 potential recombination events. Based on our findings besides point mutations, insertions and deletions, and reassortment, recombination also plays a significant role in the evolution of avian orthoreoviruses and contributes to their genetic diversity.



Phylogenetic tree based on the partial nucleotide sequences of the σC gene of chicken and turkey orthoreoviruses.

In addition to the analysis of the above described strains, in the course of the PhD studies of Dr. Bence Gál, 446 samples had been collected from turkey, chicken and duck farms in Hungary, Ukraine, Romania and Russia in 2016 in co-operation with Dr. Imre Horváth-Papp. All together we could isolate reoviruses from 113 animals. The samples were derived from healthy flocks and flocks with various clinical symptoms (runting-stunting syndrome, arthritis, etc.). The partial sequence of the σC coding gene of the strains was determined and analyzed in 47 cases. Except for 3 strains, all Hungarian isolates were distantly related to and appeared separately from the vaccine strains (S1133, 1733, 2408) on the phylogenetic trees. The complete genome sequence of 8 strains belonging to different genogroups had also been determined, but analyzing these data is under process. This great diversity of Hungarian strains might be the explanation that the currently used vaccines do not provide complete protection against the presently circulating strains in the Hungarian flocks.

In order to classify chicken orthoreoviruses, we aimed to analyze the complete nucleotide and amino acid sequence of the σC gene responsible for virus neutralization. Analysis of the σC of our strains and sequence data available from GenBank is currently in progress.

Turkey orthoreoviruses

Turkey orthoreoviruses have been described in connection with a number of diseases with various clinical symptoms, and can cause arthritis, tendonitis, myocarditis, gastrointestinal diseases, etc. Since at the beginning of our project we had very scanty information about European strains, we analyzed 3 turkey origin strains (19831M, D1246 and D1104) in co-operation with the CEVA-Phylaxia. The strains had been collected in Hungary from turkey flocks in 2009. Strain 19831M had been isolated from a 14-days-old turkey with ruffled feather, pericarditis and enteritis. D1246 and D1104 had been isolated from 35-days-old and 42-weeks-old animals. The near complete genomic sequences of the 3 strains had been analyzed. The strains showed the highest degree of similarity with each other (99.2-100 % nucleotide sequence and 98.2-100 % amino acid sequence similarity) and their genomic organization was similar to and corresponded with that of the US origin Reo/PA/Turkey 22342/13. On the phylogenetic trees, the strains formed a monophyletic group and showed close relationship with other turkey strains. In the analysis of most genomic segments, host specific evolution could be observed; the turkey, chicken and waterfowl origin reoviruses formed well-separated groups on the phylogenetic trees in case of most genomic segments. In case of the μB gene, this kind of separation could not be seen; turkey and chicken origin reoviruses and waterfowl strains belonging to the novel type formed a common group most probably due to the reassortment events that occurred in the past.

Partridge orthoreovirus

Avian orthoreoviruses have not only been detected from poultry, but also from different wild bird species. During our research program we studied the reovirus strain D1007/2008 isolated from partridge (*Perdix perdix*) in connection with sporadic mortality and clinical signs characteristic to infra-orbital sinusitis and pneumonia. With the exception of the λA, μA, μNS and σA genes, which were only distantly related to the known reoviruses of Galliformes, the other segments of the partridge reovirus strain showed similarity to chicken and turkey reovirus strains, referring to the different origin of the genomic segments and indicating that this mosaic genomic constellation is viable in partridge. The presence of chicken and turkey origin

segments in a given genome seems to refute the earlier assumption that chicken and turkey reoviruses would be members of separate species in the genus *Orthoreovirus*. Based on our results we could presume that partridges and other wild birds can play a reservoir role for poultry reoviruses. Our data suggested that chicken or turkey origin reoviruses can successfully infect and adapt to other bird species following host transmission events. Co-infection with homologous strains and in case of successful replication with heterologous strains might contribute to the generation of novel reassortant viruses with different biological properties.

Pheasant orthoreoviruses

In the course of our project we could determine the complete genome sequences of 3 pheasant isolates. Extensive genetic diversity could be observed between the studied pheasant strains. Strain 18769 exhibited the highest degree of similarity with the D1007/2008 partridge reovirus strain and appeared on a separate branch only in case of the μNS coding gene. The 216/2015 strain was analyzed in collaboration with Dr. János Gál (University of Veterinary Medicine, Budapest). Strain 216/2015 was isolated from a diarrheal pheasant flock. The strain was similar to chicken reoviruses. The third strain, D1996, was only distantly related to known avian orthoreovirus strains.

In vitro reassortment studies

In addition to the analysis of naturally occurring reassortant strains *in vitro* studies had also been performed. *In vitro* co-infection with the chicken vaccine strain S1133 (Nobilis reo) and T1781 isolated previously in Hungary had been performed in LMH and BHK-21 cell lines. Plaque purification was performed to identify potential reassortants. Following propagation of the selected plaques, RNA purification was carried out. A real-time PCR assay had been developed for most genomic segments to detect the reassortant strains. We could identify the origin of the genomic segments based on the different melting points of the generated PCR products. For the M1 and S3 segments specific primers had to be designed. 12 reassortant strains had been identified using the above described methods. We isolated 8 reassortants from BHK cell line: BHK35, BHK36, BHK39, BHK42, BHK43, BHK45, BHK52 and BHK70; and 4 reassortants from LMH cell line: LMH119, LMH120, LMH123 and LMH124. 4 reassortant strains had been randomly selected and subjected to ion torrent sequencing. Data from next generation sequencing is currently processed.

To investigate the evolutionary mechanisms of reptilian orthoreoviruses co-infection studies had been performed with the distantly related 47/02 and CH1197/96 reptilian orthoreovirus strains in viper heart cell line, but generation of reassortant strains could not be proved.

Reverse genetics

Reverse genetics and protein synthesis related work had to be delayed because our research team received the necessary GMO permissions only on July 13, 2016. However, we did not get permission to create complete viruses. We designed a plasmid-based reverse genetics system for selected genomic segments of the S1133 avian and 47/02 reptilian orthoreovirus strains, which was based on the adaptation of the previously developed reverse

genetics system for mammalian orthoreoviruses. For cloning the genomic segments pTWV228 low copy plasmid was purchased. The reverse genetics system was driven by a plasmid-encoded T7 RNA polymerase, therefore pCAG-T7 plasmid construct was purchased and tested on VH-2 and BHK-21 cell lines. The reoviral S1 genomic segments of S1133 and 47/02 were integrated between the upstream T7 RNA polymerase promoter and the downstream incorporated self-cleaving ribozyme from hepatitis delta virus (HDV) and T7 terminator sequences. mRNA synthesis from the prepared clones was checked by applying an *in vitro* transcription kit. Effectivity of the transcription driven by the T7 polymerase enzyme and the subsequent HDV ribozyme self-cleavage was determined by real-time RT-PCR. Based on our results, only 15 to 23 % of the mRNA product remained uncleaved. Though mRNA synthesis proved to be effective by applying *in vitro* transcription kits, no protein production could be detected using *in vitro* translation method.

Publishing the results

Results of the research project have been published in 9 international and 1 Hungarian scientific journal, the cumulative impact factor was 26. Based on the accumulated data we plan to prepare and submit further 3-4 manuscripts in 2018-2019.

Katalin Ihász successfully defended her thesis, and later she has finished her PhD studies (Molecular characterization of reptilian RNA viruses, including reptilian reoviruses) at the Doctoral School of the Pannon University. Dr. Bence Gál and Renáta Kugler has finished their PhD studies at the Doctoral School of SZIE ÁOTK. B. Gál studied the diversity, evolution and taxonomy of avian orthoreoviruses, while R. Kugler conducted her research project about the molecular characterization of reptilian orthoreoviruses, respectively.