Summary of Phd thesis

Genetic diversity of avian and reptilian origin reoviruses

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Table of contents

1. Introduction

Members of the family *Reoviridae* are double-stranded RNA viruses infecting a wide range of hosts. The genus *Orthoreovirus* belonging to the family *Reoviridae*, subfamily *Spinareovirinae* was classified into seven species at the time we started our work. Reoviruses isolated from reptiles were considered as members of the species *Reptilian orthoreovirus* (RRV), while reoviruses of bird origin were classified into the species *Avian orthoreovirus* (ARV).

Although reoviruses are commonly identified in reptiles and associated with different symptoms of the respiratory tract and central nervous system, their pathogenic role is not clear. Reoviruses of birds are frequently detected and isolated in association with infections causing significant economic losses for the poultry industry. The best defined reovirus-associated diseases are the viral arthritis-tenosynovitis in chicken and turkey, and the syndrome of waterfowl characterised by tenosynovitis and spleno- and hepatomegaly with necrotic foci.

Major evolutionary mechanisms of orthoreoviruses are i) point mutations generated by the viral RNA-

dependent RNA polymerase lacking proofreading activity, ii) intrasegmental insertions, deletions and duplications, iii) reassortment of cognate genome segments between members of an *Orthoreovirus* species.

The currently used classification of orthoreoviruses is based on biologic properties and genetic characteristics. In the genus *Orthoreovirus* specific species demarcation criteria have been defined based on sequence identity values. >75% nucleotide (nt) sequence identity between homologous genes is the cut-off value for most genomic segments to classify virus strains into the same species, and <60% nt sequence identity is considered to be the cut-off value to demarcate viruses into different virus species. In case of the outer capsid proteins, where the amino acid (aa) sequence identity is >55% indicates that orthoreoviruses belong to the same species, while <35% similarity is used to classify virus strains into different species. For the core proteins higher values have been defined, >85% for strains in the same species and <65% for strains belonging to different species. For the non-structural proteins cut-off values have not been clearly defined

Various next-generation sequencing (NGS) methods can provide opportunity to collect complete genomic

sequence data of the circulating virus strains, broadening our knowledge about genetic diversity and phylogenetic relationships of the viruses causing infections.

The widespread use of NGS methods can help to develop novel diagnostic methods and more effective vaccines to reduce economic losses. In this context, the main objective of our studies was to collect and characterise complete genomic sequence data of reoviruses originating from different host species.

2. Objectives

The aim of our study was to uncover the genetic diversity of reoviruses isolated from different reptile, pheasant and waterfowl species:

1. to collect genomic sequence data applying NGS technology;
2. to analyse the collected data and discover the genome structure, genetic diversity, and phylogenetic relationships of the studied strains. Thus, we could gain more information about the evolutionary mechanisms of the reoviruses which could be further studied in '*in vitro*' circumstances in cell cultures;
3. discovering the phylogenetic relationships of the studied reovirus groups. Comparison of different classical and novel waterfowl reovirus strains of European and Asian origin, and in the case of pheasant reoviruses, with reoviruses isolated from gallinaceous birds.

3. Materials and methods

Virus strains and cell lines

We analysed five orthoreovirus strains derived from different avian species. Strains D2533/4/1-10 and D2533/6/1-10 were isolated in 2014 from the bursa Fabricii of Pekin ducklings (*Anas platyrhyncos domestica*) originated from a flock in Germany. Strain Reo/HUN/DuckDV/2019 was isolated from Pekin ducks from a Hungarian flock in 2019. Strain D1996/2/1 was isolated in 2012 from the gizzard and bursa Fabricii of a pheasant (*Phasianus colchicus)* originating from a Hungarian pheasant farm. Strain Reo/HUN/Pheasant/216/2015 was isolated in 2015 from the pooled stool sample of a Hungarian pheasant flock.

We also analysed seven reovirus strains isolated from different exotic reptilian species. Two strains were isolated in Germany: IBD26/00 derived from a *Boa constrictor* and 55/02 from a carpet python (*Morelia spilota*). Strain CH1197/96 was isolated from a spur-tighed tortoise (*Testudo graeca*) in Switzerland. Four strains were isolated in Hungary: KP3 from a ball python (*Python regius*), 2013/12 from a Schneider's skink (*Eumeces schneideri*), 2013/54 from a green iguana

(*Iguana iguana*) and 2013/47 from an unknown snake species.

Avian origin reovirus strains were propagated on LMH (ATCC CRL-2117) cell line, reptile origin strains were propagated on VH-2 (ATCC CCL-140), IgH-2 (ATCC CCL-108) and TH-1 (ATCC CCL-50) cell lines. VH-2 cells were used in the coinfection assay.

Molecular methods

Viral nucleic acid was purified using TRI Reagent. Reverse transcription (RT) was performed with the viral RNA using the oligonucleotide FR26RV-N consisting a 3' random hexamer tag. cDNA was amplified in PCR using the FR20RV oligonucleotide. The PCR product was run in agarose gel, products between the size of 200-2000 base pairs were excised and extracted. Libraries were prepared and whole genome sequencing was performed on IonTorrent and Illumina NGS platforms. To obtain the terminal sequences a modified 5' and 3' RACE method was applied.

Coinfection assay was carried out to study the reassortment of cognate genomic segments of RRVs. VH-2 cells were coinfected with 47/02 and CH1197/96 strains. After cytopathic effect was evident the cell culture

was passaged four times. Viral RNA was extracted from the individual strains that were plaque-purified from the passaged cell culture. The possible reassortment events were detected by one-step RT-PCR. The origin of each segment was identified by high resolution melt (HRM) analysis based on the melting temperature of the product amplified from the segment. The RT-PCR and HRM analysis were carried out on a StepOne Plus Real-Time PCR system, the raw data were analysed using the StepOne Software v2.3 and High Resolution Melt (HRM) Software v2.0.

Bioinformatics

CLC Genomics Workbench v7 was used to clean and assemble NGS data. Sanger sequencing reads were edited by BioEdit and Geneious Prime softwares. Geneious Prime and AliView softwares were used to assemble NGS contigs and Sanger reads. BLASTn and BLASTx algorithms were used to identify homologous genes among sequences deposited in GenBank.

Codon-based multiple alignments were obtained using Geneious Prime and TranslatorX softwares. Phylogenetic analysis was performed, and sequence identity values were calculated using the MEGA6

package. Gene-specific substitution models were evaluated, and the best-fit models were selected based on the Bayesian information criterion. Maximum-likelihood trees were generated, and tree topologies were validated by bootstrap analysis (100). Nt and aa sequence distances were calculated using *p*-distance method.

4. Results and discussion

We determined the complete genome sequence of four avian reovirus strains (three Pekin duckling and one pheasant origin) and the complete coding sequence of one pheasant origin strain using next generation and Sanger sequencing methods. We also determined the complete genome sequence of seven reovirus strains isolated from different exotic reptilian species. The genome size and genomic organization of the studied strains were similar to that of the previously described reoviruses. The complete genomes were ~23-24 kbp in size and consisted of ten segments, encoding 11 proteins in case of RRVs and 12 proteins in case of ARVs.

Waterfowl reoviruses

Based on their genetic properties WRVs are divided into two categories, the classical and the novel WRVs. In novel WRVs the S1 segment, similarly to other ARVs is tricistronic encoding the σC cell attachment fiber protein, the p17 protein responsible for the regulation of different nuclear and cytoplasmic processes and the p10 protein. The classical WRVs possess bicistronic S4 encoding the σC protein and the p10 protein. The p10 proteins of the

classical and novel WRVs are non-homologous, the latter belongs to the fusion-associated small transmembrane (FAST) proteins responsible for giant cell formation in cell cultures. Although the strains examined in the recent study possessed tricistronic S1 segment indicating that these belong to the novel WRVs, the analysis of the complete genome sequences revealed more complex relations.

The strains D2533/6/1-10 and Reo/HUN/DuckDV/2019 shared moderate to low similarity with other ARVs. The nt and aa identity values were between 38,06-72,38% and 25,63-84,79%, respectively. These values mostly fell either below the cut-off value of the species demarcation criteria for different species or into the so-called 'grey-zone', between the two cut-off values of species demarcation. Contrary to this, the identity values were all above the species demarcation criteria when comparing with strain Ych, which was isolated in 2019 from a Pekin duck in China (86,48-95,01% nt, 94,08-99,21% aa identity). Phylogenetic analysis of individual genes confirmed the strong relationship between D2533/6/1-10, Reo/HUN/DuckDV/2019 and Ych strains; they always

appeared on the same monophyletic branch, distinctly from other ARVs.

Strain D2533/4/1-10 showed moderate to high nt and aa identities with non-waterfowl ARVs (40,09-78,56% nt, 31,16-94,65% aa identity). The highest nt/aa identity values were observed in comparison with classical and novel WRVs of European and Chinese origin (45,91-94,27% nt, 42,21-98,69% aa identity). In case of 6 genes (λA, λB, λC, μA, μNS and σNS) pairwise distance analyses revealed the highest nt sequence identity values with classical WRVs, while 3 genes (μB, σB, and σC) were most similar to novel WRV strains. Sequence analyses of the σA gene revealed similar nt identity values with strains belonging to both types of WRVs. These findings were confirmed by the topology of the phylogenetic trees. In the σC phylogeny, D2533/4/1-10 clustered together with the novel WRVs but appeared on a separate branch indicating that this segment was most likely acquired from a divergent reovirus strain of a heterologous host species. On the phylogenetic tree based on the σC gene classical and novel WRVs compose well separated groups, but they are more closely related to each other than to the ARVs of gallinaceous birds. The bicistronic S4 segment of classical WRVs might have been derived from the

tricistronic S1 segment of novel WRVs by losing the FAST protein.

According to our analyses D2533/4/1-10 proved to be a triple reassortant strain, which supposedly obtained its λA, λB, λC, μA, μNS and σNS protein coding genomic segments from classical WRVs, while μB, σA and σB protein coding segments from novel WRVs. The origin of the S1 segment encoding the σC protein remained unidentifiable. Strains D2533/6/1-10 and Reo/HUN/DuckDV/2019 might have been acquired from unknown host (presumably not domesticated bird) species; these viruses were able to cross the species barrier and successfully replicated in waterfowls. Our data indicated greater genetic distance in comparison with other ARVs: the identity values were lower than expected when any two members within the same orthoreovirus species were compared, therefore the classification of these strains remained undetermined.

Our data reaffirmed that reassortment has a relevant role in the evolution of waterfowl orthoreoviruses. Detecting novel variants of WRVs, evolved by reassortment or cross-species virus transmission, broadens our knowledge of the diversity of orthoreoviruses in waterfowl.

Pheasant reoviruses

Nt and aa sequences of the coding regions of Reo/HUN/Pheasant/216/2015 showed the highest sequence identity values with reference chicken, turkey, and partridge reovirus strains (65,11-94,46% nt, 59,09-99,22% aa identity) and were much lower when compared with WRVs (39,43-77,53% nt, 29,80-95,90% aa identity). In case of the λB, λC, σB genes Reo/HUN/Pheasant/216/2015 clustered with chicken origin reoviruses while in case of σC and σNS genes turkey origin reoviruses were its closest relatives. In case of the λA, μA, μB, μNS and σA genes it grouped together with reovirus strains from gallinaceous birds, but appeared on a separate branch in the group. On the phylogenetic tree based on μB gene Reo/HUN/Pheasant/216/2015 did not cluster with any ARVs used in our calculations.

According to our data Reo/HUN/Pheasant/216/2015 obtained its genomic structure through several reassortment events with reoviruses of gallinaceous birds. Our findings reaffirmed that turkey and chicken origin reoviruses belong to the same species, as reassortment could occur between these two groups of reoviruses.

Strain D1996/2/1 showed only low similarity with any of the currently known reoviruses isolated from birds. In comparison with ARVs from different hosts low identity values were observed (38,09-71,04% nt, 30,30-80,82% aa). Although slightly higher identity values were seen in the comparison with the members of the newly described *Neoavian orthoreovirus* (NeARV) species (45,64-72,72% nt, 37,37-88,46% aa identity), these values also remained below the cut-off value of species demarcation of identical species. The closest relatives of D1996/2/1 were two NeARV strains: Pycno-1 isolated from a brown-eared bulbul in Japan and TVAV isolated from a hooded crow in Finland. In phylogenies performed with all genomic segments, D1996/2/1 appeared in a common branch with these strains but was only distantly related to other known orthoreoviruses.

The identity values between D1996/2/1 and other ARVs were lower than expected when any two members within the same orthoreovirus species were compared. Although on the phylogenetic trees grouped monophyletically with the NeARV strains, D1996/2/1 could not be classified into the species NeARV neither. Because of the limited amount of reovirus sequences available from wild animals the exact origin of D1996/2/1

remained unknown. The high level of nt sequence identity found in the 5’ UTR sequences with other avian and neoavian orthoreovirus strains may suggest the avian origin of this strain.

Reassortment of the cognate genomic segments of ARVs deriving from different hosts has a significant role in the evolution of these viruses. The emerging of novel variants in domestic fowl might raise new challenges in the prevention and vaccination against reovirus infections. All pheasant origin orthoreovirus strains included in our analyses belong to different genogroups indicating the high diversity of orthoreoviruses that are capable of replication in pheasants. Game birds kept in semi-intensive or extensive farming conditions might be more vulnerable to different infections and possible reservoirs in the spreading of reoviruses and other pathogens.

Reptilian orthoreoviruses

Reptile origin reovirus strains showed only low similarity when compared with other orthoreovirus species (28,72-64,12% nt, 11,72-66,88% aa identity values). Phylogenetic analysis revealed four well separated lineages within the genus *Orthoreovirus*,

reaffirming previous findings: i) the three serotypes of the classical mammalian orthoreoviruses (Jones, Lang, Dearing); ii) avian and neoavian orthoreoviruses with those of bat origin: Nelson Bay and Pulau reoviruses; iii) reptilian and testudine orthoreoviruses along with Baboon, Broome and Mahlapitsi reoviruses and iv) Piscine orthoreoviruses.

Within the group of the studied reptilian orthoreoviruses three different lineages could be observed: i) strains 2013/54 (green iguana, *Iguana iguana*) and 55/02 (carpet python, *Morelia spilota*) grouped with reference RRV strain 47/02 (bush viper, *Atheris squamigera*); ii) strains 2013/12 (Schneider's skink, *Eumeces schneideri*), 2013/47 (unknown snake species), KP3 (ball python, *Python regius*) and IBD26/00 (*Boa constrictor*) composed one group; iii) strain CH1197/96 (spur-tighed tortoise, *Testudo graeca*) clustered separately on each phylogenetic trees. Most of the sequence identity values between strains belonging to the same lineage reached the demarcation criteria for identical species. In the comparison of the strains grouping on different branches, the results were more diverse: most of the identity values fell between the cut-off values of the species demarcation criteria, and in some

cases below the cut-off value for distinct species. The structure of the bicistronic S1 segment was slightly different in the two squamata origin reovirus groups: the strains 2013/12, 2013/47, IBD26/00 and KP3 possess two overlapping ORFs on the S1 segment, while on the S1 segment of strains 47/02, 55/02 and 2013/54 the ORFs are non-overlapping.

Pronounced genetic distances could be observed between strain CH1197/96 isolated from spur-tighed tortoise and other ortoreoviruses including the reptilian origin strains. In comparison with other RRVs the nt and aa identity values mostly fell either below the cut-off value for different species or into the so-called 'grey-zone', between the two cut-off values of species demarcation. This might evolved due to the distant genetic relationship between the squamata and chelonian hosts. 70 isolates from the coinfection assay were tested but no reassortant isolate were find.

Our findings indicate a very early diversification of homologous viruses within their recognized reptile hosts and co-evolution of these viruses. Whole genome sequencing and phylogenetic analyses permitted to separate squamata origin orthoreoviruses into two major phylogenetic branches whose members are

characterized by high similarities within genogroups and moderate similarities among genogroups. The unsuccessful reassortment assay, the different hosts and the low identity values indicating that the tortoise reovirus could be the first representative of a novel reptile origin *Orthoreovirus* species.

5. New scientific results

1. We determined and analysed the whole genome sequence of the first representatives of novel WRV strains isolated from Pekin ducklings in Europe. The characterization of one strain revealed mosaic-like genomic composition, with classical and novel WRV origin segments, confirming the significance of reassortment in the evolution of WRVs. Two strains isolated from Pekin ducklings proved to be only distantly related to previously described European and Asian waterfowl origin orthoreoviruses thus these might be the first representatives of a new genogroup within the WRVs. Our findings provided further information about the high genetic diversity and evolutionary mechanisms of waterfowl origin orthoreoviruses.

2. We determined and analysed the complete coding sequences of the first representatives of ARVs isolated from pheasants in Europe. The genome of one strain consisted of turkey, chicken and partridge origin segments reaffirming the significance of reassortment in the evolution of ARVs derived from gallinaceous birds. One pheasant origin strain showed moderate similarity

with members of the species ARV and NeARV, so this virus might be the first representative of a new *Orthoreovirus* species. Our findings revealed high genetic diversity of reoviruses that are capable of replication in pheasants and the possible reservoir role of the pheasants in the spreading of ARVs.

3. We analysed the phylogenetic relationships of the RRVs based on whole genome sequence data of reoviruses isolated from different exotic reptile species. Our data and analyses permitted to separate squamata origin orthoreoviruses into two genogroups. One chelonian origin strain proved to be distantly related to squamata origin reptilian reoviruses thus our group suggested its classification as a separate species. The chelonian reovirus strain was accepted by the ICTV as the first representative of the novel *Testudine orthoreovirus* species.

4. Publications in peer-reviewed journals related to the thesis

Kugler, R., Marschang, R., Ihász, K., Lengyel, G., Jakab, F., Bányai, K., Farkas, S. (2016) **Whole genome characterization of a chelonian orthoreovirus strain identifies significant genetic diversity and may classify reptile orthoreoviruses into distinct species.** Virus Research. 215. 94–98. 2016. http://doi.org/10.1016/j.virusres.2016.02.005

Farkas, S., Varga-Kugler, R., Marton, S., Lengyel, G., Palya, V., Bányai, K.: **Genomic sequence and phylogenetic analyses of two novel orthoreovirus strains isolated from Pekin ducks in 2014 in Germany.** Virus Research. 257. 57–62. 2018. http://doi.org/10.1016/j.virusres.2018.09.001

Varga-Kugler, R., Palya, V., Farkas, S., Bányai, K.: **Reovirus infections of waterfowls.** Magyar Állatorvosok Lapja. 142. 387-396. 2020.