Summary of Phd thesis

Genetic diversity of poultry reoviruses in Eastern Central Europe

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1. Introduction

In the United States, a group of researchers observed a marked increase in cases of tenosynovitis caused by avian reoviruses in poultry populations beginning in the 2010s. Laboratory investigations revealed that the causative reoviruses belong to previously unidentified genetic clusters. It is hypothesized that these novel variants may have overcome the immunity conferred by conventional commercial vaccines. This discovery has sparked renewed scientific interest in avian reoviruses, as evidenced by the growing number of publications on the topic. Numerous research groups are now working to better understand the relationship between the genetic diversity of reoviruses and the clinical manifestation of the disease.

Avian reoviruses are of significant concern due to their economic, veterinary, and vaccinological implications. In Hungary, the poultry industry is a major contributor to the national GDP, generating an annual value of 500–550 billion HUF. Diseases caused by reoviruses—such as tenosynovitis and enteritis—reduce production efficiency, increase mortality and culling rates, and result in substantial financial losses. Infections predominantly

affect young broiler chickens but can also impact other poultry species. Clinical signs include arthritis, diarrhea, growth retardation, and mortality. The virus is highly resistant to environmental conditions and remains infectious for extended periods. It spreads rapidly and can be transmitted across continents via live birds or hatching eggs.

The virus evolves rapidly, and emerging variants may evade immunity provided by existing vaccines. The genetic variability of the σC protein is particularly critical for vaccine efficacy, as this protein mediates cell attachment and elicits immune responses. Accurate identification of genetic clusters and epitopes is essential for diagnostics and vaccine development. The use of farm-specific autogenous vaccines is becoming more common, although their development is time- and resource-intensive. Continuous research is required due to the virus's genetic variability.

Avian reoviruses exhibit diverse evolutionary mechanisms, including:

Point mutations, primarily driven by the RNA-dependent RNA polymerase enzyme;

Insertions, deletions, and duplications within genome segments, contributing to the emergence of novel variants;

Reassortment, which occurs when a host cell is co-infected by multiple viruses, allowing homologous segments to be exchanged.

Molecular methods and genetic characteristics are increasingly used for virus classification, gradually replacing classical virological techniques. According to current taxonomic guidelines, two virus strains are classified within the same species if:

- Their nucleotide sequence identity exceeds 75%;
- Their core protein amino acid sequences are at least 85% identical;
- Their outer capsid protein sequences share at least 55% identity.

Conversely, strains are classified as different species if:

- Nucleotide identity is below 60%;
- Core protein amino acid identity is less than 65%;
- Outer capsid protein identity is under 35%.

The widespread adoption of next-generation sequencing technologies enables the complete sequencing of viral genomes, providing detailed insights

into genetic diversity and evolutionary relationships. These methods support the development of more accurate diagnostic tools and more effective vaccines.

The primary objective of our study was to perform a detailed analysis of the σC gene or complete genome sequences of reoviruses isolated from chicken and turkey flocks, and to investigate the correlation between genetic diversity and clinical disease presentation.

2. Objectives

During my research, I aimed to gain a comprehensive understanding of the occurrence and genetic characteristics of avian reoviruses in Central and Eastern Europe. My study was structured around the investigation of the following hypotheses:

1. In poultry flocks in Central and Eastern Europe, reoviruses can be isolated at a higher rate from animals exhibiting symptoms characteristic of reovirus infection compared to asymptomatic animals.
2. Based on the σC gene, chicken reoviruses circulating in Central and Eastern Europe can be classified into genetic clusters I. - V.
3. The nucleotide composition of the S1 gene segment encoding the σC protein is identical in reovirus strains isolated from chickens and turkeys in Central and Eastern Europe.
4. The nucleotide composition of the S1 gene segment encoding the σC protein in reovirus strains isolated from chickens and turkeys in Central and Eastern Europe determines the clinical manifestation of reovirus-induced disease.

3. Materials and methods

Samlpe collection and preparation

Sample collection was conducted over the course of one year, partly during necropsies performed on poultry farms and partly through sample collection and submission by collaborating veterinarians. As a result, samples originated not only from Hungary but also from Romania, Russia, and Ukraine. The study included various tissue samples (e.g., small intestine, cecal lymph node, tendon, tendon sheath, synovium), and to a lesser extent, samples from other organs (e.g., liver, kidney, trachea).

The investigation did not have a diagnostic purpose; therefore, comprehensive laboratory testing was not performed. During the collection process, detailed background information was also recorded (e.g., animal species, age, medical history, vaccination status, and place of origin). The samples were delivered to the laboratory under refrigerated conditions, where they were identified and subsequently processed.

Virusisolation

The samples were individually homogenized using sterile phosphate-buffered saline solution (PBS) and a sterile metal bead. This was followed by centrifugation at 3000 × g for 2 minutes at 4 °C. The supernatant was then filtered through a 0.45 µm pore-size polyethersulfone (PES) membrane filter. The resulting filtrate was used as the inoculum.

Virus isolation was performed using a hepatocellular carcinoma cell line derived from domestic chicken (LMH, ATCC CRL-2117). The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and penicillin/streptomycin antibiotics, and incubated at 37 °C in a thermostat with 5% CO_2.

The inoculum was applied to the cells using a four-step, tenfold serial dilution. Cytopathic effect (CPE) was monitored daily under a microscope. Giant cell formation, characteristic of avian reovirus (ARV), typically appeared on days 5–6. In the absence of CPE, observation continued for up to 7 days.

Samples showing CPE were marked as positive (CPE+). If no CPE was observed after 7 days, blind passages were performed. After three consecutive

negative blind passages, the sample was considered ARV-negative and was discarded.

Molecular methods

Nucleic acid extraction was performed from the available samples using TRI reagent. The extracted viral RNA was then reverse transcribed into complementary DNA (cDNA) using a reverse transcription (RT) reaction. The resulting cDNA was amplified via polymerase chain reaction (PCR) using a chicken reovirus σC gene-specific PCR assay.

Amplification was carried out using the following primer pairs:

- S1_all_F3 (Forward): 5′-GATACTSTCNTTGACTTCGA-3′
- S1_all_R2 (Reverse): 5′-TCGATGCCSGTACGCACGGT-3′

These primers target an approximately 900-nucleotide region (positions 674 to 1603) of the S1 segment of the S1133 vaccine strain genome.

PCR products were verified by agarose gel electrophoresis. Products within the 200–2000 base pair (bp) range were purified using a gel extraction method. The amplified and purified cDNA was then used for library

preparation, followed by Sanger sequencing and whole genome sequencing using Illumina next-generation sequencing platforms.

Data analysis

Electropherograms obtained from Sanger sequencing were read and edited using BioEdit and Geneious Prime software. The resulting sequences were aligned with the next-generation sequencing (NGS) data using Geneious Prime and AliView.

Homologous gene searches were performed in the GenBank database using the BLASTN or BLASTX algorithms.

Codon-based multiple sequence alignments were carried out using the online alignment tool integrated in Geneious Prime. Phylogenetic analyses and sequence identity calculations were conducted using the MEGA X software suite. The best-fitting substitution model for phylogenetic tree reconstruction was selected based on the Bayesian Information Criterion (BIC).

Phylogenetic trees were constructed using the maximum likelihood method, and the reliability of the resulting trees was assessed via bootstrap analysis with 100 replicates.

Average nucleotide and amino acid distances between sequences were calculated using SDT v1.2 software.

4. Results and discussion

Turkey reoviruses (TARV)

Turkey reoviruses (TARV) have been known since the 1980s. Although they typically cause mild disease, their impact can be exacerbated by secondary infections. In the 2010s, novel and atypical genotypes emerged in the United States, associated with increased morbidity and mortality. A study conducted in Hungary in 2016 aimed to genetically characterize local TARV strains and compare them with strains from other countries.

Samples were collected from turkeys in three Hungarian counties—Békés, Vas, and Veszprém—in 2016. The sample set included a total of 32 specimens. In Veszprém County, tendon samples (n=10) were collected from a flock of 14-day-old male growers. Lameness and uneven growth were observed in 10% of the flock, while mortality remained within normal limits. Necropsy revealed serous-fibrinous-hemorrhagic inflammation of the hock joint, with no other notable lesions. Reovirus was successfully isolated from 6 of the tendon samples (60%).

In Békés County, lameness and uneven growth were observed in an 8-week-old meat turkey flock, affecting approximately 20% of the birds, equally across both

sexes. Inflammation of the hock joint was noted, but no other lesions were identified. Tendon (n=10) and intestinal (n=5) samples were collected from deceased birds, with one tendon and one intestinal sample testing positive for reovirus. Samples from Vas County were excluded from analysis due to suspected contamination at the slaughterhouse.

Genetic analysis revealed that seven isolates belonged to Cluster 2—six from the second Veszprém case and one from Békés—while one isolate from Békés was classified into Cluster 3. Notably, all virus strains isolated from tendons (n=7) were assigned to Cluster 2, whereas the single intestinal isolate (n=1) belonged to Cluster 3.

Further nucleotide-based comparisons showed:

- 100% identity among Cluster 2 strains in this study,
- >99% identity with a TARV strain isolated in Hungary in 2009 (Cluster 2),
- 95.8–97.2% identity with TARV strains from the United States (Cluster 2),
- 84% identity with Hungarian partridge-derived reoviruses,
- 93.5% identity with Hungarian pheasant-derived reoviruses (Cluster 2).

The study observed low genetic variability, particularly in the gene encoding the σC protein, which is responsible for antigen binding and cell attachment. This genetic stability is likely due to the absence of vaccination and the limited geographic spread of the virus. Some strains were classified into new genetic clusters, suggesting that TARV genetic diversity may be greater than previously assumed.

Phylogenetic analyses also revealed reassortment events, especially involving the μB gene, which showed evidence of gene mixing between chicken- and turkey-derived strains. This raises the possibility of interspecies gene exchange, although evidence for this in Hungary remains limited.

The genetic similarity among Hungarian TARV strains suggests a common origin. However, further studies involving international samples are needed to clarify the exact lineage relationships. This research highlights the importance of international collaboration to better understand the evolution and spread of TARV.

Chicken reoviruses (ARV)

Sample collection was conducted throughout 2016 with the assistance of farm veterinarians and consulting

veterinary experts. A total of 391 samples were collected from 38 poultry farms across four countries: Hungary (n=346, 31 farms), Romania (n=36, 4 farms), Ukraine (n=6, 2 farms), and Russia (n=3, 1 farm). In Hungary, samples were obtained from the counties of Bács-Kiskun (n=20), Baranya (n=53), Békés (n=6), Borsod-Abaúj-Zemplén (n=40), Csongrád (n=9), Győr-Moson-Sopron (n=30), Hajdú-Bihar (n=44), Szabolcs-Szatmár-Bereg (n=94), and Veszprém (n=50), representing nearly the entire country.

Samples originated from broiler flocks (n=248), broiler breeder flocks (n=137), and commercial layer flocks (n=6). Clinical background information was provided by collaborating veterinarians, and samples were categorized based on observed symptoms: lameness, runting-stunting syndrome, uneven growth, diarrhea, peritonitis, increased mortality, and clinically healthy (i.e., no ARV-specific symptoms). Approximately half of the samples came from healthy birds (n=197), while the other half were from symptomatic flocks (n=194). The most common clinical signs were: Runting-stunting syndrome (n=74), Lameness (n=52), Uneven growth (n=43), Diarrhea (n=5), Peritonitis (n=6), Increased mortality (n=4) or Unknown clinical status (n=10).

ARV detection rates were highest in flocks with: Runting-stunting syndrome (28/74 = 38%), Uneven growth (15/43 = 35%) and Increased mortality (1/4 = 25%).

Lower detection was observed in flocks with lameness (3/49 = 6%). Infectious reovirus was found in 18% of samples from clinically healthy flocks (35/197).

Samples were primarily collected from the intestine (n=318) and tendon (n=57), reflecting the two main clinical forms of ARV infection. Additional samples came from the proventriculus (n=6), bursa of Fabricius (n=5), trachea (n=2), kidney (n=2), and liver (n=1).

Cytopathic effect (CPE), characterized by giant cell formation typical of ARV infection, was observed in 106 samples, while 285 samples were negative. At least one reovirus isolate was obtained from each country:

Hungary: 87/346 positive

Romania: 17/36 positive

Ukraine: 1/6 positive

Russia: 1/3 positive

CPE-positive samples were primarily from intestinal (n=103) and proventricular (n=3) tissues. No CPE was observed in LMH cells inoculated with tendon, trachea, bursa, kidney, or liver samples.

RT-PCR targeting the σC gene was performed on a subset of CPE-positive samples to confirm results. In 77.4% of CPE-positive cases, RT-PCR was not repeated, especially if the flock had already been tested.

Confirmed ARV cases were common in flocks with typical symptoms (47/184 = 26%), but surprisingly high in asymptomatic flocks as well (35/197 = 18%). Virus strains from both symptomatic and asymptomatic flocks were distributed across genetic clusters II, III, IV, and V. A significant difference was found between these groups (chi-square test = 16.9, p = 0.00004).

Sampled flocks ranged in age from day-old chicks to 51-week-old birds, with 71% (277/391) being six weeks old or younger. In this age group, ARV was isolated in 68 cases, representing 83% of positive samples. No virus was isolated from birds younger than 10 days.

Sequencing of a 768-nucleotide region of the σC gene was performed on 72 CPE-positive isolates. After identifying closely related sequences in GenBank using BLAST, pairwise identity and phylogenetic analyses were conducted. The isolates were classified into five genetic clusters (I–V). Vaccine strains S1133, 1733, and 2408 belong to Cluster I, showing only moderate sequence

identity with the isolates in this study (nt: 49.7–74.5%; aa: 44.3–74.6%).

Sample collection from foreign countries was supported by local experts. Diagnostics relied on virus isolation and RT-PCR. Due to methodological limitations, not all ARV-positive samples yielded isolates. Interestingly, reoviruses from healthy flocks were genetically similar to those from diseased flocks, indicating potential risk.

Most samples were from the digestive tract. Although ARV was detected in the intestines of lame birds, it was not found in tendon samples, contrary to other studies—possibly due to timing or technical factors.

Hungarian ARV isolates showed considerable genetic diversity, falling into five genotypes, mainly Clusters II and IV. Genetic similarity with strains from neighboring countries (Romania, Ukraine, Russia) may reflect trade connections. Whole genome sequencing could help clarify reassortment events.

No clear correlation was found between clinical symptoms and genetic clusters, suggesting that σC-based genotyping alone is insufficient to explain pathogenicity. Future research using reverse genetics systems may enhance understanding of reoviruses and

support the development of more effective vaccines. Multivalent and region-specific vaccines may offer the most effective protection.

5. Hypothesis testing

1. In poultry flocks in Central and Eastern Europe, reoviruses can be isolated at a significantly higher rate from animals exhibiting symptoms characteristic of reovirus infection compared to asymptomatic animals.
   > False
2. Based on the σC gene, chicken reoviruses circulating in Central and Eastern Europe can be classified into genetic clusters I. - V.
   > True
3. The nucleotide composition of the S1 gene segment encoding the σC protein is identical in reovirus strains isolated from chickens and turkeys in Central and Eastern Europe.
   > True in chicken and false in turkey
4. The nucleotide composition of the S1 gene segment encoding the σC protein in reovirus strains isolated from chickens and turkeys in Central and Eastern Europe determines the clinical manifestation of reovirus-induced disease.
   > False

6. New scientific results

This is the first comprehensive study assessing reovirus infection in poultry flocks in Hungary and Central-Eastern Europe, extending to the clinical presentation, genetic diversity of reoviruses, and the relationship between these factors.

We detected reovirus strains with nearly identical σC gene sequences from the same broiler farm six years apart, suggesting that reoviruses may persist on a farm for extended periods without significant changes in the σC protein, which is responsible for inducing immunity.

In chickens, only one isolate from the proventriculus was classified into genetic Cluster III, despite the examination of several hundred intestinal samples. This unusual finding has not been previously reported and raises the question of whether Cluster III ARVs exhibit organ tropism—an area that warrants further investigation.

Our results indicate that turkey-derived reoviruses in Hungary are genetically identical regardless of geographic origin, suggesting a common source for circulating TARV strains in the country.

We also isolated and described, for the first time, a turkey-derived TARV strain belonging to Cluster III. Previously, only chicken-derived reoviruses had been assigned to this cluster, indicating a likely host species jump.

Additionally, we observed for the first time gene transfer between chicken- and turkey-derived strains in the μB protein, suggesting interspecies reassortment.

Reovirus strains from unvaccinated broiler flocks showed greater genetic variability compared to those from vaccinated flocks. Further studies are needed to understand how different vaccination strategies influence the selection and emergence of new viral strains.

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

Farkas S. L., Marton S., Dandár E., Kugler R., Gál B., Jakab F., Bálint Á., Kecskeméti S., Bányai K. (2016) **Lineage diversification, homo- and heterologous reassortment and recombination shape the evolution of chicken orthoreoviruses.** Sci. Rep. 6, 36960; doi: 10.1038/srep36960.

Gál B., Farkas Sz., Bányai K. (2021). **Csirkék reovírus-fertőzései. Irodalmi összefoglaló.** Magyar Állatorvosok Lapja 143. évf. 2021. február. 95–106.

Gál B., Varga-Kugler R., Ihász K., Kaszab E., Domán M., Farkas S. L., Bányai K. (2023). **Marked Genotype Diversity among Reoviruses Isolated from Chicken in Selected East-Central European Countries.** Animals 2023, 13, 2137. https://doi.org/10.3390/ ani13132137

Gál B., Varga-Kugler R., Ihász K., Kaszab E., Farkas S. L., Marton S., Martella V., Bányai K. (2023). **A Snapshot on the Genomic Epidemiology of Turkey Reovirus Infections, Hungary.** Animals 2023, 13, 3504. https://doi.org/10.3390/ani13223504