Etiological investigation of poultry enteric disease complexes using metagenomic approach

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Final report

In our OTKA research project, entitled „Etiological investigation of poultry enteric disease complexes using metagenomic approach" we aimed to perform metagenomic studies in order to gain more information about the composition and development of the gastrointestinal microbial community in different anatomic and functional regions using field samples originating from extensively and intensively reared chicken, and turkey poults from healthy flocks and from farms experiencing multifactorial enteric diseases. We have attempted to identify the possible virulent virus strains and pathogenic bacteria circulating in Hungarian poultry flocks and performed their genetic characterization. Several causative agents are implicated as the main causes of multifactorial enteric diseases of poultry such as viruses (rotaviruses, coronaviruses, astroviruses, reoviruses, parvoviruses, adenoviruses, picornaviruses etc.), also bacteria (*Salmonella*, *Escherichia coli*, *Clostridium*, *Campylobacter*, *Enterococcus* spp., etc.) and protozoa (*Cryptosporidium*, *Eimeria* spp.). The course of enteric diseases depends on the virulence of the pathogenic agents, coinfections, other interacting factors (management, nutrition, genetics, and hygienic measures) having effect on the immune status and susceptibility of the diseased birds. In this study, we examined the possible causes of multifactorial enteric diseases, which is essential in developing effective prevention strategies, selection of virus strains for potential mono- or multivalent vaccines.

Due to the current pandemic our studies in the 2019/2020/2021 period were mainly based on processing our sample and strain collection from the previous years, and the chicken, turkey and pheasant poult samples submitted for diagnostic pathological/histopathological examinations to NÉBIH ÁDI that already tested positive in the last few years and the broiler and breeder chicken samples collected from several farms in 2016 in Hungary. Viruses previously described as possible causative agents of enteritis have also been sequenced and characterized. Samples were collected from turkey poults to study the development of the gastrointestinal microbial community.

Methods

Metagenomic analysis of viral and bacterial communities was performed using different approaches and data was evaluated to optimize sample preparation methods to avoid false results and conclusions. We have selected 5 Gram+ and 5 Gram- bacterium strains from our culture collection to see which of the commonly used methods is most suitable for downstream processing. DNA extraction was performed with a silica membrane column-based method and a magnetic bead-based method. PCR amplification of the variable regions of 16S RNA demonstrated that both extraction methods work well in our hands.

To examine the effectiveness of sample preparation for viral metagenomics we set up a methodical study to test whether coronaviruses, parvoviruses and reoviruses spiked in sterile filtered stool specimens can be efficiently detected by random primed RT-PCR coupled with deep sequencing of amplified DNA fragments. In this process we first demonstrated that filtering and nuclease treatment of cell culture samples positive for these viruses, respectively, very efficiently decreased the amount of host origin DNA/RNA; up to 80-90% of host origin

nucleic acids could be eliminated by this method when comparing plain nucleic acid extraction without preceding filtering and nuclease treatment.

Farm chicken

During the first year we collected samples taken weekly from healthy farm chicken to examine the development and complexity of the microbiome in comparison with chicken raised in intensive farming conditions. These samples included pieces of the small intestine, caecum, and large intestine. Each organ was homogenized, and nucleic acids were extracted separately, processed by using unbiased amplification and metagenomic sequencing. In total, 54 samples (taken from 18 chickens) were processed, and each sample was handled individually. An average 1.65 Million (range, 0.62 to 2.41 Million) sequence reads were generated for each specimens irrespectively of the gut fraction (with other words, approximately 5 million reads were generated for each chicken). A total of 54 samples have been evaluated by basic bioinformatic tools thus far. In general, a combined prevalence of 0.7 to 6.2 percent of reads could be assigned to bacteria, fungi and viruses (including phages). Based on sequence read distribution the most common bacteria were *Chlamydia psittaci*, followed by various *Proteobacteria* (*Paracoccus versutus*), *Actinobacteria*, *Bacilli* and *Clostridia*. Samples collected on the first week contained *Clostridia* only in the caecum, while these appeared from the second week in the large intestines too in different ratios. On the 6th week members of *Bacteriodetes* could be detected in all sections of the intestines. The most common viral reads were from families *Baculoviridae*, followed by *Herpesviridae*, *Iridoviridae*, bacteriophages including *Microviridae* and *Myoviridae*. Pigeon paramyxovirus 1 was a notable member of pathogenic viruses (although this virus needs to undergo some specific genomic mutations to be able to cause disease in chicks); some of the remainder viruses were likely of dietary origin. Among fungi, that constituted <<0.1% of sequence reads we detected members of *Ascomycota* (*Aspergillus niger*, *Botrytis cinerea*) and *Basidiomycota* (*Moniliophthora roreri*, *Melampsora larici-populina*). On the 6th week avian coronavirus (Infectious bronchitis virus) could be also detected without any clinical signs in the large intestine samples.

Technically, it is of note that the percentages of viral reads were considerably lower than we expected from data generated in previous year when spiked stool samples were processed using the same protocol. We assume that tissue and cell debris (with their DNA and RNA content) were much more difficult to remove from organ samples than from stool samples, therefore host-origin nucleic acids were more abundant. We also hypothesize that viral infection in chickens raised in the backyard are less heavily affected by viral infections.

We also tested a few stool samples we had in our freezers. Among 12 chicken, 7 turkey and 3 pheasant origin fecal samples, each collected from diarrheic animals, we detected rotaviruses, reoviruses, astroviruses and avisiviruses. Rotaviruses (RVs) were detected in all three host animals. Among rotaviruses four species, RVA, RVD, RVF and RVG were identified by viral metagenomics. Reoviruses were detected in turkey and pheasant samples, whereas astroviruses and avisiviruses (a newly described genus within *Picornaviridae*) were detected only in turkeys. The proportion of viral reads were greater in these specimens (up to 31% of reads), but these samples were taken from stool samples of diseased animals in which the proportion of viral particles can be very high (eg. for rotaviruses 10 to 9 or even 10 to 10 viral particles per gram stool).

Multifactorial enteric diseases

Diagnosis of multifactorial enteric diseases is challenging due to the non-specific clinical signs and lesions, detection and/or isolation of enteric viruses and bacteria is difficult, the pathogenic

role of viruses/bacteria is still unknown, interaction between the pathogenic agents is not fully understood, and opportunistic viruses/bacteria might also play role in the pathogenesis of multifactorial enteric diseases. In this study the intestinal virome of PEMS-affected turkey, pheasants and chicken were analysed. Most of the viruses detected in our study have already been associated with enteric diseases but using sequence-independent amplification and deep sequencing on a high throughput platform, coinfections, as well as novel and highly divergent viruses have also been revealed in the gut of poultry. Novel viral genome sequences were determined and analysed.

Metagenomic analysis of chicken samples

Chicken origin samples (Fig 1.) contained food origin viruses, members of *Phycodnaviridae* (Coccolithovirus), *Baculoviridae* and different phages. Pathogenic viruses appeared in single or mixed infections. For example, in the samples, herpesviruses could be detected, either alone (I/5/9), or in a mixed infection with RVG (II/2/7). Parvoviruses also caused a mixed infection with avian orthoreoviruses and RVG. Picornaviruses were detected in one sample (22966).

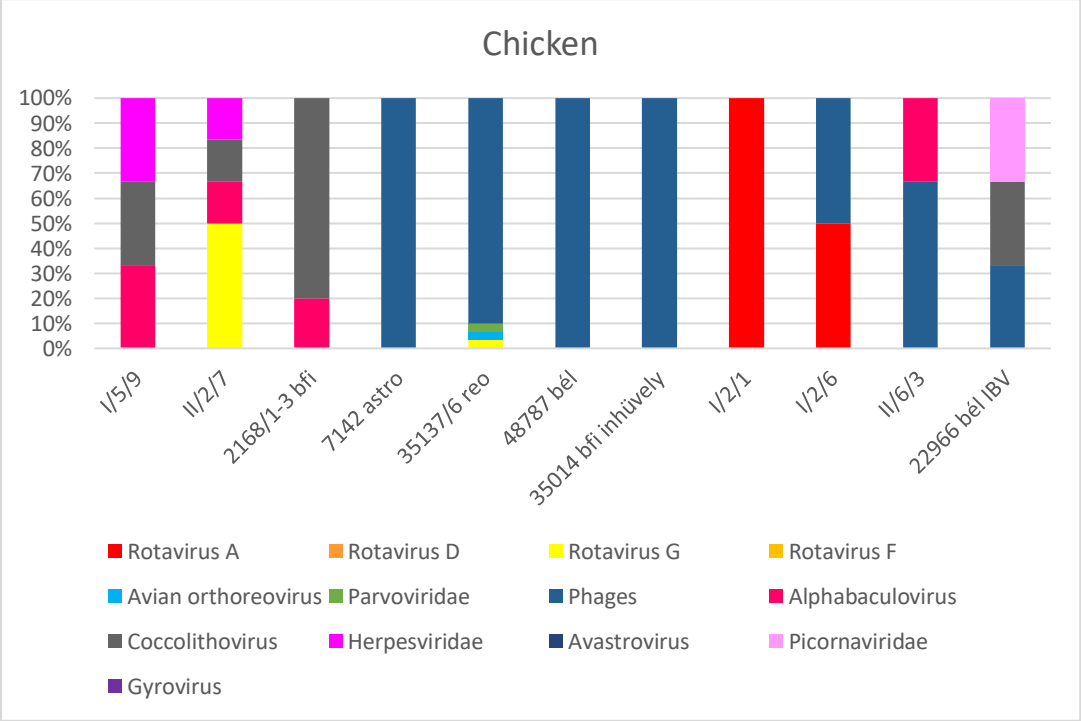


Figure 1. Distribution of viral reads in the chicken gut samples.

Metagenomic analysis of turkey samples

In turkey samples (Fig 2.) food origin viruses, members of *Phycodnaviridae* (Coccolithovirus) *Baculoviridae* and different phages could be detected. In the PEMS samples *Rotavirus A* could be detected in 15.5% of the samples, while RVD in 3.4 %, and RVF in 5.2 % of the samples, respectively. The most abundant rotavirus species was RVG which could be detected in 34.5 % of the samples, as a single infection or in a mixed infection with RVA or with herpesviruses. Herpesviruses occurred almost in the third of the samples (32.7 %) in many cases in mixed infections with rotaviruses belonging to different genotypes, gyroviruses or avastroviruses. Picornaviruses were detected in three cases, gyroviruses in one sample, parvoviruses and avastroviruses were found in four-four samples. Interestingly, no avian orthoreoviruses could

be recognized by this method. The above-mentioned viruses have already been recognized as possible viral agents contributing to the development of PEMS.

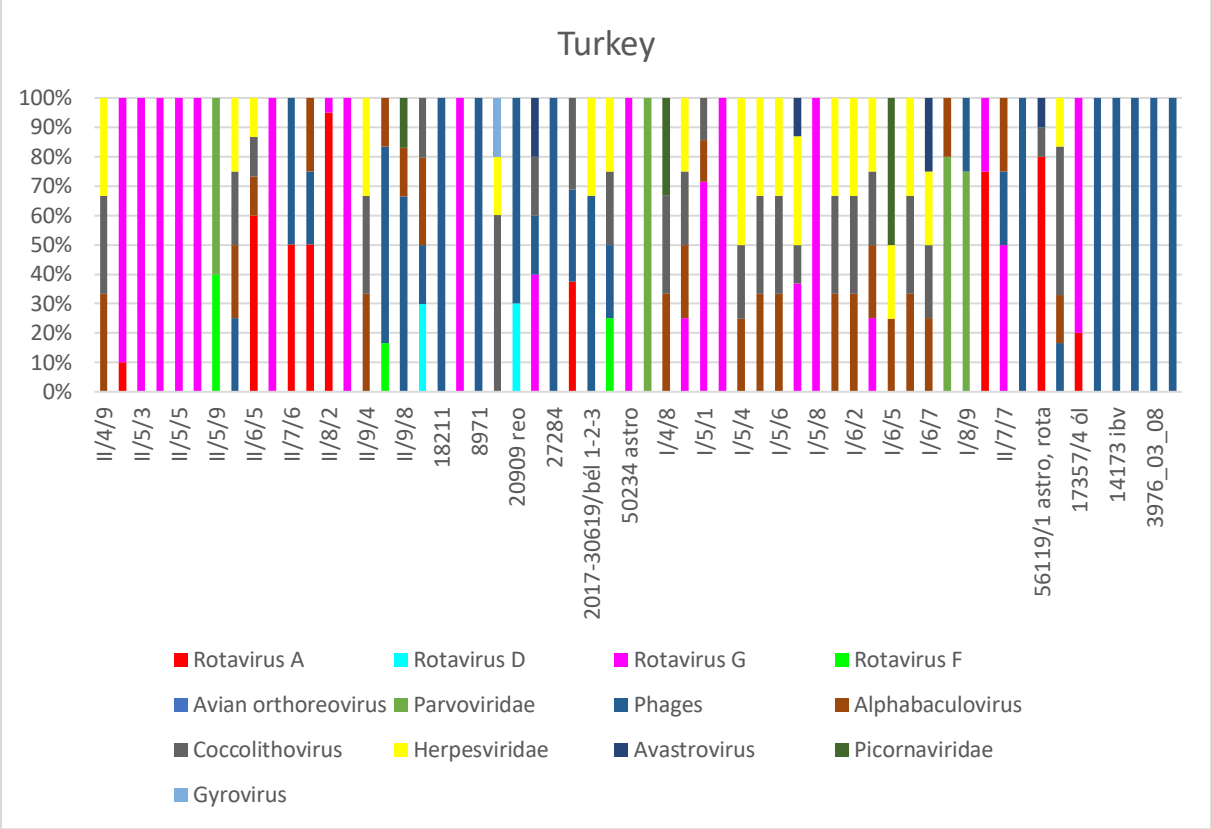


Figure 2. Distribution of viral reads in the turkey gut samples.

Metagenomic analysis of pheasant samples

Pheasants (*Phasianus colchicus*) are among the most popular game bird species, millions of pheasants are bred and reared in the world for sporting purposes. Game birds at younger age are usually farmed semi-intensively and later extensively before being released to the wild. Enteric diseases affecting intensively housed poultry can also cause considerable losses for game bird breeders. There is still limited information available in the literature about the etiology of enteric disease syndromes in these species, further studies are needed to discover which pathogenic agents play role in the development of the clinical signs and lesions. Game bird species are well-known reservoirs of pathogenic agents of poultry. Infected by viruses of different origin provides opportunity for recombination and reassortment leading to the development of novel viral strains with different characteristics. To discover the genetic diversity of viruses circulating in these species, samples originating from diseased birds were processed and analysed. Early identification of novel variants infecting poultry and related species might help to develop preventive measures.

Six samples were submitted for metagenomic analysis. In the pheasant samples (Fig 3.) food origin viruses, members of *Phycodnaviridae* (Coccolithovirus) *Baculoviridae* and different phages could be detected. Among the pathogenic viruses RVA could be detected in one sample (II/1/5), herpesvirus could be detected in three samples (I/6/3, I/8/8, I/9/8), while a mixed infection, gyroviral and parvoviral infection could be observed in sample II/9/3.

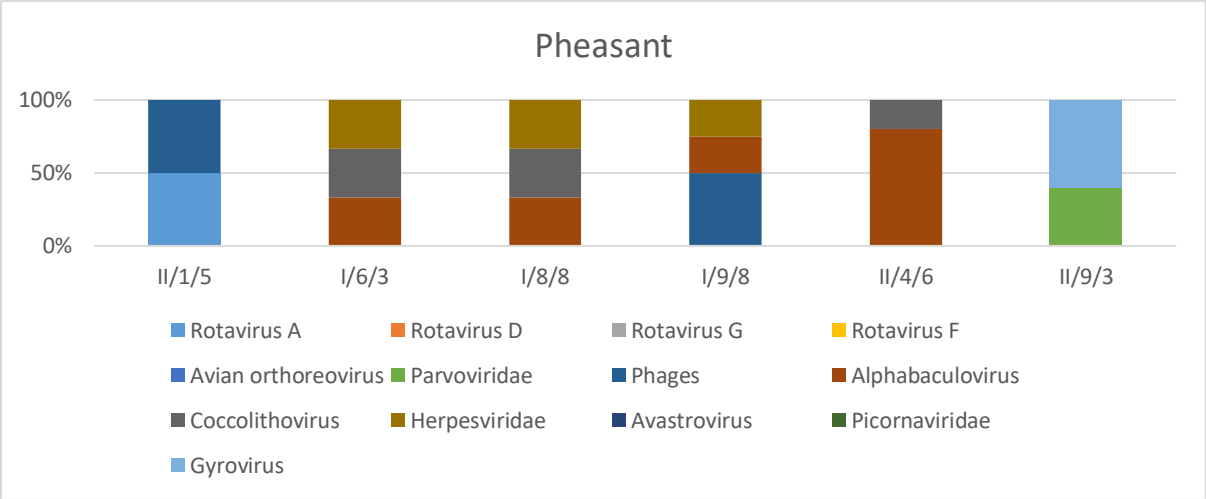


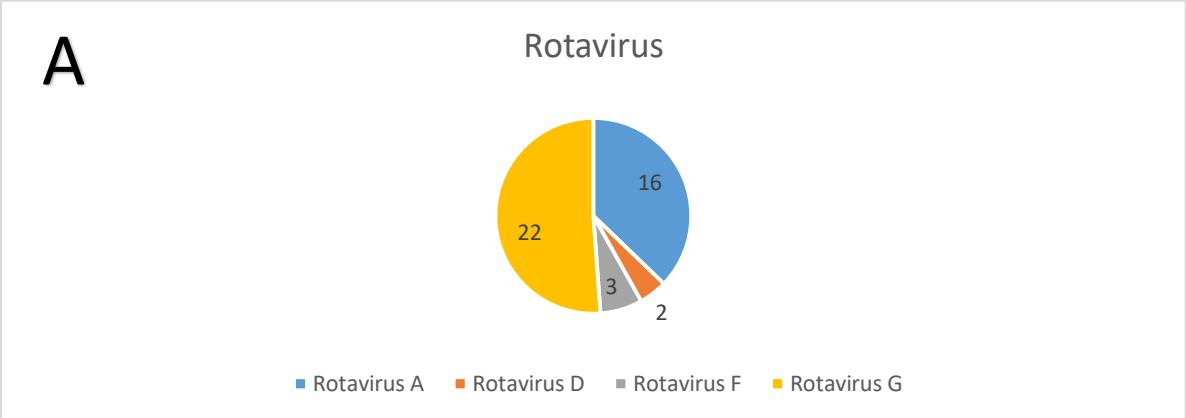
Figure 3. Distribution of viral reads in the pheasant gut samples.

Rotaviruses in PEMS

(Manuscript in preparation)

RVA, RVD, RVF, and RVG are known to naturally infect birds; RVD, RVF and RVG are found solely in poultry. RVA and RVD have been the most frequently detected members of the genus, members of these two genotypes have been associated most often with diseases in several avian species, while group F and G avian rotaviruses have been reported only occasionally.

In chicken RVA and RVG were detected in 2-2 samples (I/2/1, I/2/6 and II/2/7, 35137/6). In pheasant RVA was detected in sample II/1/5 (Fig. 4). In turkey RVs were detected in 39 cases. Interestingly RVGs were detected most frequently, in the 51.1 % of the samples. The second most abundant genotype was the RVA, diagnosed in 37.2 % of the samples. RVD and RVF occurred only in few samples. RVs showed simultaneous infection with a variety of other enteric viruses, including other RV genotypes, herpesviruses, avastroviruses, orthoreoviruses.



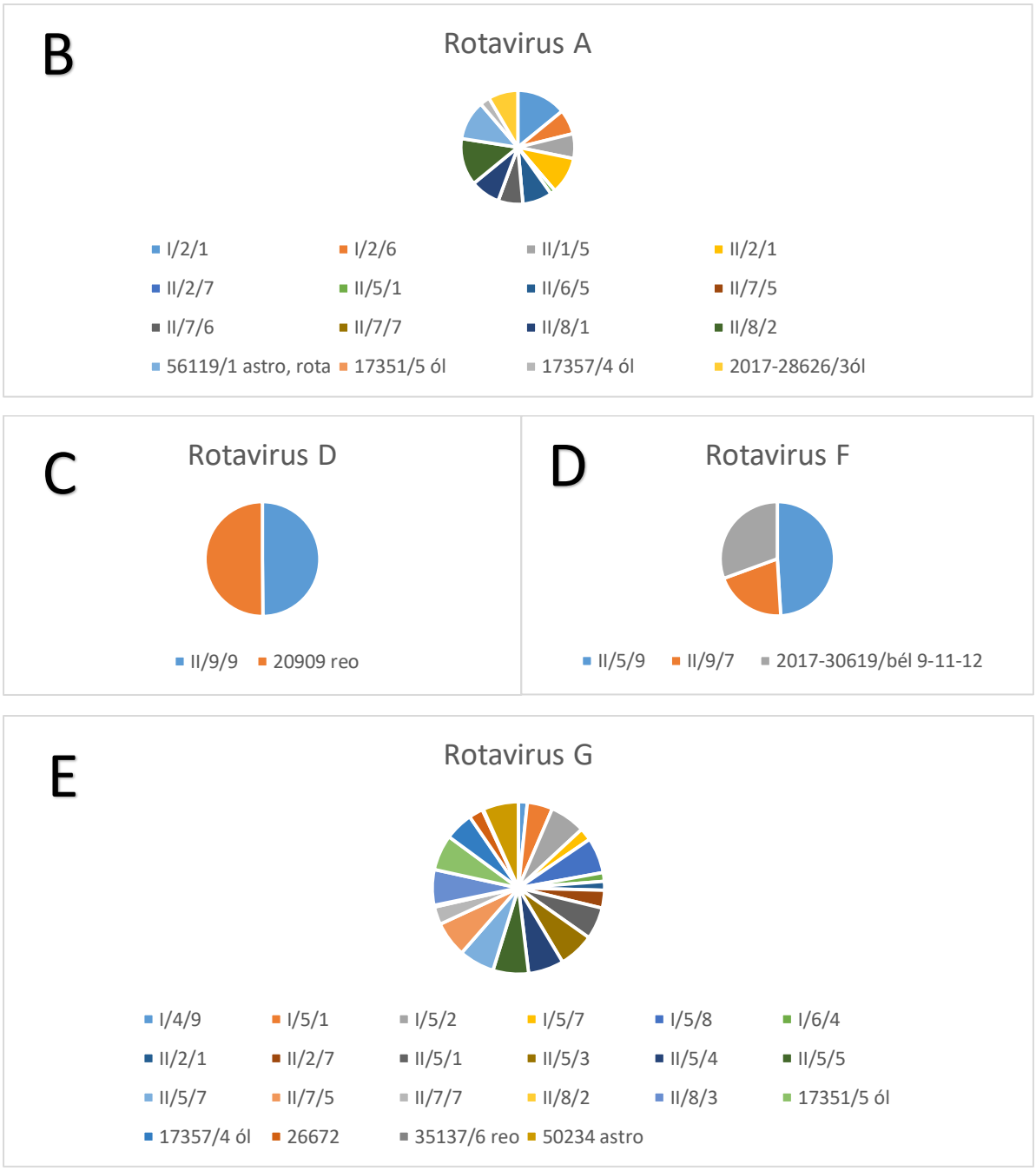


Figure 4 (A-E). Prevalence and distribution of rotavirus species in the PEMS samples.

Genomic characterization of avian and neoavian orthoreoviruses detected in pheasants

(Virus Research, IF 3.303)

To study the genomic features and evolutionary history of pheasant reoviruses originating different geographic areas the complete/coding-complete genomic sequences of two Hungarian reovirus strains, D1996/2/1 and Reo/HUN/Pheasant/216/2015, have been determined. Both strains were isolated from birds showing clinical signs and lesions during necropsy. Strain D1996/2/1 was isolated in 2012 from a pheasant flock with increased mortality, with gizzard erosion and internal bleeding. Reo/HUN/Pheasant/216/2015 was identified in pooled stool samples from young poults with ruffled feathers, poor appetite, increased water consumption,

and diarrhoea. In addition to Reo/HUN/Pheasant/216/2015, a rotavirus strain, RVA/pheasant-wt/HUN/216/2015/G23P[37] which was detected by metagenomic analyses and described in another study (Gál et al., 2016) might have also played role in causing the symptoms. Phylogenetic analyses showed the Hungarian isolates were only distantly related to the pheasant strain detected in the United States. Reo/HUN/Pheasant/216/2015 showed a mosaic genomic composition, and shared genetic relationship with turkey, partridge, and chicken reoviruses, and in case of two genes the origin could not be determined. The other isolate, D1996/2/1 did not form a common group with the currently known members of the species *Avian orthoreovirus*, it might represent a novel orthoreovirus species or a new genogroup within the newly accepted species, *Neoavian orthoreovirus*. Clustering of this strain needs further studies. Based on our study high genetic diversity among pheasant reoviruses could be observed.

Marked antigenic diversity identified in pheasant Rotavirus A strains

(Manuscript in preparation)

Rotavirus infection has been detected in intensively housed chicken and turkey, additionally in ducks, pheasants, pigeons, and wild-living bird species. RVs of birds have often been described in connection with enteritis, decreased feed conversion rate and consequential reduced weight gain, flock uniformity. RVs also play role in multifactorial diseases, such as runting-stunting syndrome in chicken and poult enteritis syndrome of turkeys. Disease in pheasant flocks with similar clinical appearance has also been detected. RVA strains collected between 2005 and 2015 were selected for analyses. Antigenic combinations were determined in case of four strains (Phe-17655Hun, Phe-14958Hun, and Phe-19109Hun), and complete genomic characterization was performed in case of two strains (Phe-14246Hun and Phe-18769Hun) to broaden our knowledge about the genetic and antigenic diversity of RVA strains circulating in pheasant flocks in Hungary. 3 G (G19, G22, G23) and 4 P type (P30, P31, P35, P37) specificities could be detected in 4 combinations. The strains selected for whole genome sequencing showed conserved constellation of backbone gene genotypes (I4-R4-C4-M4-A16-N10/N4-T4-E4-H4).

Remarkable antigenic diversity the pheasant RVAs could be observed. Mosaic genomic composition of these viruses suggests previous reassortment event between turkey, chicken, and pheasant origin strains. In one sample two RVA strains could be detected simultaneously indicating that a number of variants may co-circulate in rearing farms. These factors contribute to the difficulty in vaccine development, as the future vaccine should protect against a multiple antigen types.

Novel gyrovirus in a common pheasant (*Phasianus colchicus*)

(Accepted for publication in Archives of Virology, IF 2.574)

Pooled organ samples (intestines, brain, heart, liver, spleen) of a pheasant showing the clinical signs of poult enteritis and mortality syndrome, collected in Hungary in 2017, was processed and metagenomic analyses was performed. Analysing the data revealed the presence of a gyrovirus in the sample. The genome of the pheasant-associated gyrovirus (PAGyV) was 2353 nt long and three putative genes coding the VP1, VP2 and VP3 proteins could be detected. The genome organization was similar to that of the other gyroviruses. Sequence motifs characteristic to gyroviruses could be identified in both the coding region and the non-translated genomic region of the PAGyV genome. The VP1 of PAGyV showed only 67.6% pairwise nt identity with reference sequences and appeared separately on the phylogenetic tree. Based on the

recently determined species demarcation criteria (pairwise identity values <69% for the VP1 coding gene), PAGyV belongs to a putative novel species, tentatively named *Gyrovirus phaco 1* (initial letters of the scientific name of the host species is used), within the genus *Gyrovirus*, family *Anelloviridae*. There is no information about the host range of PAGyV, further studies are needed to explore the susceptible species.

Novel chaphamaparvoviruses in turkeys and in a common pheasant (*Phasianus colchicus*)

(Manuscript in preparation)

Members of the genus *Chaphamaparvovirus* (subfamily *Hamaparvovirinae*, *Parvoviridae*) have been discovered recently in the faecal materials or various tissue samples of chickens, turkeys, peafowl, rats, pigs, dogs, cats, parrots, straw-coloured fruit bat, common vampire bat, etc. Metagenomic studies aiming samples associated with PEMS revealed the presence of chaphamaparvoviruses in three samples originating from turkey (I/8/9, II/5/9) and a pheasant (II/9/3). The two turkey and the pheasant strains showed the highest aa sequence similarity, 66.82 %, 66.97 %, and 65.54 %, respectively with *Cygnus atratus* chaphamaparvovirus. The turkey strains were most closely related to each other, showing 97.93 % aa similarity; sequence similarity values were lower when compared with the pheasant strain, 64.45 % in case of I/8/9 and 64.75 % in case of II/5/9, respectively. On the phylogenetic tree of the NS1 protein sequences the turkey and the pheasant strains appeared on two monophyletic branches, distinctly from the previously known chaphamaparvoviruses (Fig. 5). Based on the aa sequence identity values (<85 %) the Hungarian strains belong to the genus *Chaphamaparvovirus* but appear to represent two novel species within the genus *Chaphamaparvovirus* and family *Parvoviridae*.

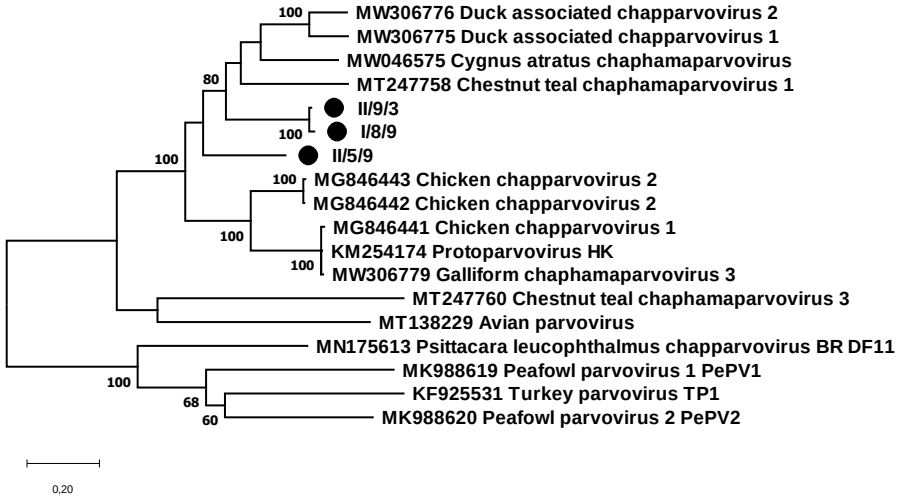


Figure 5. The phylogenetic tree shows the possible evolutionary relationship of representative avian parvoviruses. The maximum likelihood tree (100 bootstrap replicates) was constructed using the amino acid sequences of partial non-structural protein 1 (NS1) by MEGA-X software with the best fitted JTT+G+I model. The numbers near each node represent the bootstrap support. Substitution per site is represented by the scale bar.