Nucleic acid-based detection and epidemiological investigations of enteric RNA viruses associated with central nervous system infections and diarrhea in porcine and human samples

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1. Introduction

Pigs are hosts and reservoirs for numerous viral pathogens (>70) [1], including several viruses that can infect humans (spillover/host change) in some cases. These viruses include certain porcine astroviruses (family *Astroviridae*) and porcine noroviruses and sapoviruses (*Caliciviridae*) associated with diarrhea. In addition, there are several porcine viruses whose zoonotic potential has not been studied/is unknown, such as porcine teschoviruses and porcine sapeloviruses of the family *Picornaviridae*. During our research related to the Doctoral Student Scholarship of Cooperative Doctoral Program, we were focusing on the viral infections affecting two organ systems, the central nervous system (CNS) and the intestinal system.

The most common viral infections causing CNS infections and encephalomyelitis in pigs include the picornaviruses with positive sense, single-stranded RNA genomes (+ssRNA), such as porcine sapelo- (PSV) and teschoviruses (PTV), and the astroviruses, more particularly porcine astrovirus type 3 (PoAsV3) [2], which have played a central role in our research.

The **PSV** strains are currently belong to two known genotypes (PSV-1, -2) of species *Sapelovirus anglia* of genus *Sapelovirus*. These viruses have been associated with several diseases in pigs, including respiratory infections and CNS-related symptoms such as paralysis and paraplegia, but they are also frequently detectable in fecal samples from asymptomatic animals [3]. There is only limited data available on the prevalence of PSV in Hungary, especially in respiratory samples, and to our knowledge, the presence of these viruses has not yet been examined in human samples from patients with central nervous system symptoms.

Porcine teschoviruses (PTVs) of the genus ***Teschovirus,*** family *Picornaviridae* belong to two species and nearly two hundred strains [4]. Some PTV types can be associated with different clinical symptoms in pigs, such as diarrhea and respiratory diseases, and diseases with CNS involvements sometimes with high mortality rates [2]. PTVs can usually be detected directly from fecal samples, although some PTVs could also be present in respiratory samples as well [2]. Pigs are the only known hosts of PTVs,

however (or because of this) their presence have not been specifically investigated in other species, including humans.

The **astroviruses**, which also have +ssRNA genomes, are genetically one of the most diverse and species-rich groups of known viruses [5]. The surface of mature virions is mainly composed of the immunodominant VP27 and VP25 capsid proteins, which are used as antigens in serological studies of specific IgG/IgM/IgA-based immune responses [5]. Porcine astrovirus type 3 (PoAstV3), which plays an important role in our research and causes severe paraplegia/paralysis in pigs, belongs to genus *Mamastrovirus*, species MAstV-22 of the human-mink-ovine (HMO) phylogenetic clade [6].

Our second main research project was related to **digestive diseases**. Several viruses could play a role in the often etiologically complex enteric diseases of pigs, such as sapoviruses (genus *Sapovirus*), and to a lesser extent the recently discovered valoviruses (genus *Valovirus*) and the noroviruses (genus *Norovirus*), which are also important from a human health perspective, and all of them belonging to the *Caliciviridae* family. Due to faecal-oral route as the main transmission form the above-mentioned swine caliciviruses will be referred to as swine enteric caliciviruses (SECV). These viruses were played a central role in our research. Saliva/oral fluid of pigs is a relatively widely used sample type for the detection of respiratory viruses, which to our knowledge has not been used previously for the detection of enteric viruses.

Some members of the genus ***Norovirus***, which have a +ssRNA genome containing three open reading frames (ORF1-3), can infect both porcine and human hosts [7]. Swine noroviruses (Sw-NoV) were primarily detected in enteric samples from asymptomatic or mildly diarrheic animals, with generally low prevalences at all ages. Noroviruses have a huge human medical importance as well, as they are currently the leading pathogens of acute gastroenteritis outbreaks worldwide [8]. Due to the extreme genetic diversity and frequent recombination, a dual typing system (polymerase: P-types, >60 types; capsid: G-types, >48 types) has been introduced for noroviruses [8]. Within the GII genogroup, GII.11, GII.18 and GII.19 are the dominant so-called “swine

norovirus genotypes", while the genetically closely related genotypes GII.4, GII.3 (which were also sporadically detected in porcine enteric samples) and GII.12 are the human relevant pathogens [9]. Human outbreaks are generally dominated by GII.4, but between 2014 and 2017, GII.17 and GII.2 also caused serious outbreaks. So far, only 13 complete porcine norovirus capsid sequences and 5 complete genomes are known, all belonging to "swine norovirus genotypes" [10]. Among noroviruses which were considered as enteric viruses, certain human strains may also be able to replicate in salivary glands and be spread via saliva, but their presence in the saliva/oral fluid of pigs has not yet been investigated [11].

Sapoviruses (SaV) with an extremely diverse +ssRNA genome can infect both pigs and humans. Sapoviruses are divided into 19 genogroups (GI-GXIX) and at least 52 genotypes based on the differences in VP1 capsid sequences, of which GIII is the most significant in pigs, while members of genogroups GI, GII, GIV, and GV are leading in human SaV infections [9,12]. The swine sapoviruses (Sw-SaV) of GIII genogroup contain several (>11) unnumbered genotypes [12]. Asymptomatic and diarrheic Sw-SaV infections, which can occur in outbreaks, are very common among pigs, with their prevalence being highest in the post-weaning period [9]. Sapoviruses are considered as enteric viruses, but we do not have data on their presence in other body fluids, e.g. saliva. Although large numbers of human (>3600) and porcine sapoviruses (>700) sequences are available, only a few sequences are known from Hungary, all of which are previous results of our research group [12].

The first member of the genus ***Valovirus*** were described in 2009 from enteric samples of asymptomatic fattening pigs [13]. Since then, swine valoviruses (Sw-VaV) have been detected in faecal samples of pigs older than 4 months on multiple continents [14,15]. Limited data is available on the prevalence, pathogenesis and genomic diversity of valoviruses. In the case of Sw-VaV, only one genogroup and one genotype (GI.1) are known [15]. Valoviruses have not yet been detected in Hungary, human strains/types are not known at all, and their presence in saliva/oral fluid has not yet been investigated.

Multiplex RT-qPCR-based assays suitable for the detection of the viruses included in our research projects are not known. For accurate typing of caliciviruses identified by the RT-qPCR method, it is essential to determine the sequence of at least the capsid-coding genome region, for which Sanger sequencing is most often used, which, unlike "next-generation sequencing" (NGS) methods (e.g. Illumina), is not suitable for the simultaneous detection of multiple viral variants.

2. Objectives

During our research related to the Doctoral Student Scholarship of the Cooperative Doctoral Program, we developed two hydrolysis probe-based one-step multiplex RT-qPCR assays (a swine encephalomyelitis panel and a swine enteric calicivirus panel) for the detection of selected swine viruses (PSV/PTV/PoAstV3 and Sw-SaV-GIII/Sw-NoV-GII/Sw-VaV) which could be associated with two disease types, encephalomyelitis and gastroenteritis. We also performed epidemiological investigations on various porcine and human samples with these assays.

During our research with the ***swine encephalomyelitis panel***, we sought to answer the following questions:

- Can the selected viruses be detectable, and with what prevalence, in enteric and extra-intestinal samples (tissue and nasal swabs) of domestic pigs?
    - Do PoAstV3 positive asymptomatic or encephalomyelitis animals have an IgG-based antibody response against PoAstV3?
- Can other viruses be detected in different organ/tissue samples of pigs with PoAstV3-related encephalomyelitis?
- Can the selected viruses be detected in samples from archived human encephalitis cases?

In our investigations with the swine ***enteric calicivirus panel*** and related 3'RACE-PCR and NGS pipeline, we sought to answer the following questions:

- Are the selected caliciviruses detectable, and with what prevalence, in enteric and saliva samples of pigs of different ages and health status?

- What genotypes and subtypes of caliciviruses can be detected?
- What is the rate of intra- and inter-genotype co-infections?
- Can the selected caliciviruses be detected in samples from archived human gastroenteritis cases?

3. Materials and methods

Design of oligonucleotide primers and hydrolysis probes

For the swine encephalomyelitis and swine enteric calicivirus triplex RT-qPCR panels, virus-specific primers and hydrolysis probes labeled with one of three different fluorophores (6-FAM, SUN and CY-5) were manually designed for the most conserved genomic regions. Primer/probe target sites were determined by the use of alignments generated from all the available sequences of the target viruses (PSV/PTV/PoAstV3 and Sw-SaV-GIII/Sw-NoV-GII/Sw-VaV) downloaded from GenBank.

In vitro production of viral RNA standards

To standardize the reaction conditions of the RT-qPCR reactions and to verify the efficiency of the reactions, *in vitro* produced (including the steps of plasmid ligation, transformation, plasmid isolation, linearization, T7 RNA polymerase-based RNA synthesis, Qubit 4 fluorimeter measurement) 400-600 nt long viral RNA standards were used.

RT-qPCR reaction conditions and data analyses

For the encephalomyelitis panel, the LightCycler® One-step Multiplex RNA Virus Master Mix (Roche, Switzerland), while for the enteric calicivirus panel, the Luna® Universal Probe qPCR Master Mix (New England Biolabs, Ipswich, USA) were used according to the manufacturer's recommendations. RT-qPCR reactions were run on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, USA). Data analyses were performed using Bio-Rad CFX Maestro 2.2 ver. 5.2.008.0222 (Bio-Rad Laboratories, USA) software.

Testing the analytical performances of RT-qPCR panels

For both panels, the analytical sensitivity, specificity, lower limits of detection, and intra- and inter-assay reproducibility of singleplex and tripleplex RT-qPCR assays were determined using 10-fold serial dilutions of viral RNA standards.

Samples included in the investigations

For the encephalomyelitis panel, we used faecal/rectal swab samples (n=100), tissue samples (n=62) from asymptomatic (n=92) or paraplegic (n=28) animals, and nasal swab samples (n=473) from asymptomatic animals aged 21-25 days. In case of human samples, we also tested a total of 74 archived blood and cerebrospinal fluid sample pairs from cases of encephalitis of unknown etiology.

For the swine enteric calicivirus panel, a total of 198 archived enteric (n=135 diarrheic and n=63 asymptomatic) and n=228 oral fluid/saliva samples from asymptomatic animals of different ages (which were classified into four age groups) were used. A total of 336 rectal swab samples from asymptomatic swine were also examined in a retrospective follow-up study. A total of 312 archived human faecal samples were also examined of which n=75 were from children under 10 years of age with diarrhea-associated hospitalizations, while the remaining n=237 were from children under 1 year of age with the signs of gastroenteritis of varying severity (both hospitalized and home-care).

Sample preparation and total RNA extraction

Total RNA was extracted from the enteric and tissue samples using TRI® Reagent (MRC, Cincinnati, OH, USA), while from the oral fluid samples QIAamp cador Pathogen Mini kit was used which was run on a QIAcube (Qiagen, Germany) RNA/DNA Extraction Instrument according to the manufacturer's instructions.

Reverse transcription, polymerase chain reaction and Sanger sequencing

For the 3'RACE-semi nested (sn)PCR reactions of caliciviruses, outer and inner forward primers which were identical with the forward and probe sequences of the SECV RT-qPCR panel and GoTaq® Long PCR Master Mix (Promega, USA) were used.

Sanger sequencing of the PCR products were performed using the BigDye™ Terminator v1.1 Cycle Sequencing Kit (Thermo-Fisher, USA) according to the manufacturer's protocol. The resulting products were run on an ABI 3500 Genetic Analyzer (Applied Biosystems/Hitachi, Tokyo, Japan) automated sequencer.

Next-generation sequencing and data analysis pipelines

The libraries were prepared from the products of the second PCR round of 3'RACE-snPCR of caliciviruses with the NEBNext Ultra II FS DNA Library Prep Kit (NEB, USA) and run on the NovaSeq 6000/X Plus platform (Illumina, USA) with a read depth of 1, 10, and 50 million. Geneious Prime software was used to analyze the sequence data and assemble consensus sequences.

Phylogenetic and sequence analyses

Sequence alignments were performed using Multiple Sequence Comparison by Log-Expectation (MUSCLE) or CLUSTAL 2.1 algorithms. Phylogenetic trees were constructed using MEGA ver. 11.0 software. Hierarchical cluster analyses of the VP1 nucleotide sequences were performed using a script written in the R/RStudio environment.

Recombinant PoAstV3 VP27 antigen-based western blot serological test

For the production of the recombinant PoAstV3 VP27 viral capsid protein, a pLATE 5.2 expression plasmid (Thermo-Fisher, USA) containing the genome region encoding the PoAstV3 VP27 capsid protein, an "empty" plasmid (as a negative control), and competent *Escherichia coli* BL21-Rosetta 2 cells were used. The produced soluble 6xHiS-tagged recombinant protein was purified by "immobilized metal affinity chromatography" (IMAC) on a Ni-NTA column. The purified VP27 protein and the negative control isolates were separated by SDS-PAGE, then blotted onto a nitrocellulose membrane. After blocking, 1:100 diluted porcine serum samples were incubated on the membranes, the bound IgG antibodies were labeled with anti-porcine IgG-HRPO (Rockland Immunochemicals, USA) secondary antibody and visualized by

chemiluminescence technique. The GMO authorization number of the experiment is BGMF/178-10/2020.

4. Results

4.1. Results of the investigations with the swine encephalomyelitis RT-qPCR panel

The analytical sensitivity of the swine encephalomyelitis RT-qPCR panel for PSV, PTV and PoAstV3 viruses was first tested in singleplex reactions using 10x serial dilutions of *in vitro* prepared viral RNA standards, and then in a triplex format. The lower limit of detection was determined to be around 100 copies. The intra-, and inter-assay variances of the Cq values and CV% (coefficients of variation) showed values of 0.03–4.62%, and 0.86–3.29% respectively.

In a comparative investigation of conventional screening RT-PCR and tRT-qPCR assays, the agreement between the results of the two types of experiments was 90.85% for PSV, while it was 91.55% for PTV and 93.66% for PoAstV3. PCR products from samples showing different results (either tRT-qPCR+/RT-PCR− or tRT-qPCR −/RT-PCR +) were sequenced to exclude possible false positives. The total of 3 tRT-qPCR and 11 conventional RT-PCR false positive cases (non-viral or non-sequenceable) were identified.

The presence of PSV, PTV and PoAstV3 was also investigated in nasal swab samples collected from n=473 asymptomatic pigs aged 21-25 days from 28 different domestic farms. Compared to the prevalence of PSV (7.8%) and PTV (9.9%), the proportion of positive nasal swab samples for PoAstV3 (17.8%) found to be much higher. Dual infections were identified in 1.3% (n=6 PSV/PoAstV3), 1.5% (n=7 PSV/PTV) and 2.8% (n=13 PTV/PoAstV3) of the cases, while triple infections were only identified in 0.4% of the tested animals. All three investigated viruses were found at a relatively low prevalence (<30%, mostly 1–3 positive samples/farm) in most farms (66.7–75%). None of the central nervous system samples from paraplegic pigs of unknown etiology showed RT-qPCR positivity.

Western blot-based serological assays using recombinant PoAstV3 VP27 capsid protein demonstrated the presence of anti-PoAstV3 VP27 IgG antibodies in asymptomatic piglets, but not in PoAstV3-associated encephalitic animals.

All of the archived human samples from n=74 cases of encephalitis of unknown etiology was tRT-qPCR negative.

4.2. Results of the swine enteric calicivirus (SECV) RT-qPCR panels experiments in our studies

The analytical sensitivity of the SECV RT-qPCR panel reactions for Sw-SaV-GIII/Sw-NoV-GII/Sw-VaV viruses was tested using 10x serial dilutions of *in vitro* produced viral RNA standards (1E8-1E1 copies/reaction) in both single and triplex reactions under identical reaction conditions. The lower limit of detection in singleplex and triplex RT-qPCR assays were 100 copies/reaction (except for Sw-SaV, which was 1000 copies/reaction), while the intra-, and inter assay CV% variations were ranged between 0.05 and 2.75%, and between 1.17 and 5.12%, respectively.

In our SECV tRT-qPCR-based epidemiological investigations Sw-SaV was detected in 26.26%, Sw-NoV in 2.53% and Sw-VaV in 1.52% of the enteric samples of swine, but in case of saliva Sw-NoV showed the highest prevalence (7.46%), while Sw-SaV was detected in 4.39% and Sw-VaV in 0.88% of the samples.

In case of age groups, Sw-SaV was the most frequently detected virus in both enteric and saliva samples from weaned piglets (38.58% and 6.54%, respectively). Interestingly, while all Sw-NoV positive enteric samples originated from weaned piglets, most Sw-NoV positive saliva samples (n=16/17) were collected from older, fattening pigs. A total of 7 Sw-VaV were found in enteric samples from weaned piglets (n=5), and in saliva samples from one fattening and one weaned pig. The Sw-SaV positivity of enteric samples from diarrheic animals (52.38%) were significantly higher compared to asymptomatic animals (14.07%, p-value <0,001). Dual infections were identified in a total of three enteric samples (n=2 Sw-SaV–Sw-NoV and n=1 Sw-SaV–Sw-VaV infection) from two suckling pigs and one weaned pig.

In a retrospective follow-up study a total of 336 rectal swab samples from n=42 asymptomatic pigs were tested by our SECV tRT-qPCR assay. The animals were housed together in a newly established swine housing unit and individually sampled every one or two weeks, for a total of eight times. A total of n=28/42 animals (66.67%) were positive for Sw-SaV during the study period, while Sw-NoV and Sw-VaV were not detectable. The majority of the positive samples (24/28; 85.71%) originated from animals at the age of 70 days with the positivity rate of 57.1% (24/42 animals), while the remaining 4 positive (9.5% positivity rate) was found on day 77. There were no animals that were positive at both sampling times.

A total of 312 archived **human stool samples** were tested by the SECV tRT-qPCR assay, from which neither sapovirus nor valovirus could be detected, but a total of 23 samples showed norovirus positivity.

For **typing of caliciviruses** identified by SECV tRT-qPCR assay from human enteric and porcine enteric and saliva samples and to **examine the NGS-based genotype variances**, a total of 27 Sw-SaV, 13 Sw-NoV and 4 Sw-VaV 3'RACE sn-PCR products as well as n=14 NoV products from human samples were selected for NGS sequencing (NovaSeq platform, Illumina). With reference-based mapping and *de novo* assembly-based bioinformatics analyses, a total of 77 calicivirus consensus sequences could be assembled from the obtained NGS data. In the case of sapovirus sequences, half of the examined 3'RACE-snPCR products contained at least two (maximum of 5) consensus sequences/variants. For noroviruses identified from pigs, only one consensus sequence could be assembled from most of the 3'RACE-snPCR products, while two samples contained two different NoV sequence variants. For valoviruses, only one consensus sequence was generated from each of the 3'RACE-snPCR products.

A total of 8 3'RACE-snPCR products which were previously analyzed by NGS and which contained multiple consensus sequences were also sequenced by the Sanger-method, which detected only the most common variants (the ones with the highest coverage).

All the identified **SaV VP1 sequences** show closest phylogenetic relationship to sequences from genogroup GIII, except for one, which shares a common lineage with GVII strains. The GIII sequences were located on six different phylogenetic clusters (potential genotypes).

Of the identified n=13 **swine NoV VP1** sequences, n=6 were located in the GII.18 swine norovirus cluster, while the remaining n=7 was found in the swine norovirus GII.11 lineage on the VP1 phylogenetic tree. Two main lineages were clearly observable in the GII.11 cluster, which was also supported by the results of the cluster analysis of the sequences.

In our phylogenetic investigation, n=9 of the n=16 identified **human NoV VP1** sequences clustered with human noroviruses of genotype GII.4, while the remaining sequences were located in other clusters (GII.3, GII.6, GII.17). Of the human NoV strains from hospitalized cases, n=8, n=2 and n=2 belong to GII.4, GII.3 and GII.6, including two cases of GII.3/GII.4 co-infections, respectively.

The phylogenetic analysis of our identified (n=3) and all previously known (n=10) **swine VaV VP1** sequences shows the presence of two separate lineages, where our Hungarian Sw-VaV strains originating from enteric and saliva samples located on different lineages.

5. Discussion

5.1. Discussion of the findings related to the encephalomyelitis RT-qPCR panel

In the first part of our research, a novel hydrolysis probe-based encephalomyelitis RT-qPCR panel was developed which can simultaneously and selectively detect all known porcine teschoviruses (PTV), porcine sapeloviruses (PSV) and porcine astrovirus type 3 (PoAstV3) with a relatively high sensitivity. The analytical sensitivity and specificity investigations showed optimal efficiency and specificity of the encephalomyelitis tRT-qPCR assay. The diagnostic sensitivity of the tRT-qPCR assay with previously used RT-PCR-based screening tests on archived RNA samples was also compared, where a generally higher reliability of the tRT-qPCR assay was found. After

assessing the diagnostic efficiency, an epidemiological investigation in several sample types was also conducted.

Among the samples of PoAstV3-infection-related paraplegic pigs, the PoAstV3 was also detected in intestinal samples, which indicates the previously unknown possibility of low-level fecal excretion of the virus during acute paraplegia. Beside CNS the internal organs of the paraplegic pigs were also positive for PTV and PSV, indicating a disseminated co-infections with PoAstV3, PTV and PSV in addition to PoAstV3 neuroinfection. These co-infections may have contributed to the severity of the observed disease and also indicate a reduced immune status of the affected animals, which can be supported by the absence of anti-PoAstV3 IgG antibodies PoAstV3-infected paraplegic pigs.

Of the three analyzed viruses, PoAstV3 was the most prevalent both in nasal swab samples and in the investigated farms, followed by PTV and PSV, which were detected at considerably lower prevalence. Previous nasal swab prevalence data are only available for PoAstV3, where the highest detection rate (30% positivity) was found in 3-week-old animals. Our results show that all three neurotropic viruses, especially PoAstV3, are widespread and endemic in the majority of the large-scale industrial pig farms in Hungary. The exact background of the neurotropism of AstVs is not yet known [5], however, disseminated infection and neuroinfection probably do not develop in animals with a well-developed immune system [16], as indicated by the presence of anti-PoAstV3 IgG antibodies on samples taken from asymptomatic pigs. All the examined archived human blood and CSF samples were negative for all three viruses by tRT-qPCR assay, which may indicate that these porcine-origin viruses are either not capable of causing human disease or are not widespread enough to be detected in such a small number of tested samples.

5.2. Discussion of the findings related to the swine enteric calicivirus RT-qPCR panel

In our second research project, an optimally sensitive and efficient triplex RT-qPCR-based assay, a related 3'RACE semi-nested PCR-based whole-capsid amplification protocol, and an NGS-based virus variant identification pipeline were developed, which is suitable for the sensitive, specific and simultaneous detection of the most common swine enteric caliciviruses/SECVs (swine sapovirus GIII, norovirus GII, and valovirus GI) and for capsid-based typing of the viruses/variants. The process was tested in a small epidemiological study using archived porcine enteric and oral fluid samples and archived faecal samples from human gastroenteritis cases.

The most common virus detected in archived swine enteric samples using our SECV RT-qPCR assay at both animal and farm level was Sw-SaV, followed by Sw-NoV, while Sw-VaV was detected for the first time in Hungary in two geographically distant farms. The highest positivity of the three viruses was found in enteric samples of weaned piglets. In the case of Sw-SaV, the results are in line with the literature [17], while in the case of Sw-NoV and Sw-VaV, the age-related prevalence data of the literature might be not reliable due to the limited number of related studies. Of the three viruses tested, only Sw-SaV was detected with a significantly higher prevalence in diarrheal samples (52.38% / 14.07%), which supports the enteropathogenic role of some Sw-SaV strains in certain cases [17]. In addition to numerous single infections, a small number of dual infections with SaV-NoV (n=2) and SaV-VaV (n=1) were also found, of which the SaV-VaV co-infection detected in pigs is a new finding.

A naturally occurring asymptomatic Sw-SaV outbreak was also identified by SECV tRT-qPCR in a newly established swine housing unit as a part of a retrospective follow-up investigation, where faecal shedding of the virus lasted less than 2 weeks.

The presence of all three viruses (with the dominance of Sw-NoV) were also detected for the first time in oral fluid/saliva samples of pigs. This suggests that these swine enteric caliciviruses may be able to spread in pigs in a previously unknown way, via saliva. Our results show that saliva samples can be an easily collectable and cost-effective alternatives for the investigation of not only respiratory but also enteric (calici)viruses.

To type caliciviruses and investigate genotype variance, ≈2100-2500 bp-long 3' genome regions encoding the complete capsids were amplified by 3'RACE-snPCR, and sequenced by NGS (NovaSeq, Illumina).

A total of n=77 calicivirus consensus sequences (n=45 Sw-SaV, n=13 Sw-NoV and n=3 Sw-VaV, n=16 human NoV) were identified from the NGS reads using reference mapping-based and *de novo* assembly-based approaches, including multiple (up to five-fold) co-infection variants. Cases of co-infection have been previously reported from several pig farms, although with considerably lower genotypic diversities [17].

Sanger sequencing of 3'RACE-snPCR products containing multiple variants showed that only the most dominant variant appears on Sanger electropherograms. Therefore, the use of NGS in epidemiological studies of caliciviruses is strongly recommended in both swine and human studies.

Based on the results of the phylogenetic analysis of **sapoviruses** and the accepted genotype demarcation criteria [18], all identified Sw-SaV variants belong to 7 different genotypes (n=6 GIII and n=1 GVII). These identified genotypes were commonly found in the investigated swine farms, of which several SaV variants/genotypes were co-circulating. More than half of the SaV 3'RACE-snPCR products contained at least two (up to five) phylogenetically distinct Sw-SaV variants, indicating a high level of co-infections and a significant genetic diversities of circulating SaV strains in the investigated swine farms/populations.

All n=13 **Sw-NoV** consensus sequences were derived from saliva samples, and all them belong to the "swine norovirus genotypes", more precisely genotypes GII.11 and GII.18. The distribution/occurrence of the identified genotypes is consistent with results of previous investigations [19]. Furthermore, there were several farms examined, where both Sw-NoV genotypes were detectable, which could indicate the co-circulation of these types in the given pig populations. At the beginning of our investigations, only n=13 full-length Sw-NoV VP1 capsid sequences were available, which number were doubled due to our results.

Two novel, distinct sub-lineages of Sw-NoV GII.11 strains were identified by phylogenetic analysis, where all Hungarian GII.11 strains from saliva forms a distinct sub-lineage. The separation of GII.11 strains into sub-lineages may indicate a separate intra-genotypic evolutional pattern of different NoV sub-types/variants of predominant GII.11 similar to those found among the dominating GII.4 type of human noroviruses.

The NoV strains identified from saliva samples were phylogenetically distinct from previously found enteric strains, which may suggest that enteric-origin and saliva-origin Sw-NoVs may have different tissue tropisms.

The study **Sw-VaV strains** from Hungary and Slovakia form two previously unidentified lineages in the VP1 phylogenetic tree, which were also separated into two groups by cluster analysis. So far, only n=12 full-length Sw-VaV capsids have been identified, of which only one originates from Europe [14]. All three study Sw-VaV sequences were phylogenetically distant from the Italian strains, indicating an unknown depth of genetic diversity of Sw-VaVs circulating in swine populations in this region.

Only NoV positive samples were found in the human enteric samples by the SECV tRT-qPCR assay. The prevalence data in samples from hospitalized children under 10 years of age (12%) and in patients under 1 year of age (5.9%) with gastroenteritis of varying severity (both hospitalized and home-care) are lower than the previously reported values. This may be due to nucleic acid degradation related to long-term storage of tested samples, or to the fact that our test was originally designed for swine calicivirus sequences and did not take into account human calicivirus sequences.

All n=16 human NoV consensus sequences assembled from NGS data belong to the genogroup GII, and none of them are related to swine norovirus GII types. The most common GII type was the dominant GII.4 (9/16, 56.3%), which showed the closest relationship to those of the pandemic GII.4 variants which were dominant at the time of sampling (e.g. New Orleans-2009 from 2011 samples, while Sydney-2012 from 2015/2016 samples) [20]. In addition to GII.4, other pandemic strains of GII.3, GII.6 and GII.17 were also found [20]. In more severe cases requiring hospitalization, also GII.4 dominance (8/10, 80%) was found, similar to the results of previous studies

[20,21]. The remaining two cases of acute gastroenteritis-related hospitalizations were both associated with NoV GII.6 infection, which has been also previously described in similarly severe cases. There were two gastroenteritis cases requiring hospitalization (2/14, 14.3%) in which two different GII genotypes (GII.4-GII.3 co-infection) were detected simultaneously. The presence of intra-genogroup NoV co-infections has only rarely been investigated/reported in human cases, so their frequency is unknown, and there is also no information on whether mixed NoV infection is associated with more severe symptoms, possibly requiring hospitalizations or not.

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Publications

Publications related to the Ph.D. dissertation:

1. László, Z., Pankovics, P., Reuter, G., Cságola, A., Bodó, K., Gáspár, G., ... & Boros, Á. (2022). Development and Large-Scale Testing of a Novel One-Step Triplex RT-qPCR Assay for Simultaneous Detection of "Neurotropic" Porcine Sapeloviruses, Teschoviruses (*Picornaviridae*) and Type 3 Porcine Astroviruses (*Astroviridae*) in Various Samples including Nasal Swabs. Viruses, 14(3), 513. https://www.mdpi.com/1999-4915/14/3/513.

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2. László, Z., Pankovics, P., Urbán, P., Herczeg, R., Balka, G., Igriczi, B., ... & Boros, Á. (2025). Multiple Co-Infecting Caliciviruses in Oral Fluid and Enteric Samples of Swine Detected by a Novel RT-qPCR Assay and a 3′ RACE-PCR-NGS Method. Viruses, 17(2), 193. https://www.mdpi.com/1999-4915/17/2/193.

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2. Neuroinvasive porcine astrovirus infection associated with recurrent outbreaks of encephalitis, posterior weakness and paralysis among weaned piglets (scientific poster). 12th European Symposium of Porcine Health Management (ESPHM), Budapest, April 14. 2022.

Other publications:

László, Z., Pankovics, P., Reuter, G., Cságola, A., Bálint, Á., Albert, M., Boros, Á. Multiple Types of Novel Enteric Bopiviruses (Picornaviridae) with the Possibility of Interspecies Transmission Identified from Cloven-Hoofed Domestic Livestock (Ovine, Caprine and Bovine) in Hungary. VIRUSES 13: 1: 66, 19 p. (2021)

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