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Detection of atypical porcine pestivirus in Hungary

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Table of Contents

List of Abbreviations

aa	Amino acids
APPV	Atypical porcine pestivirus
BVD	Border disease virus
BVDV	Bovine viral diarrhoea virus 1, 2
cp	Cytopathic
CSFV	Classical swine fever virus
CT	Congenital tremor
DNA	Deoxyribose Nucleic acid
ER	Endoplasmic reticulum
FFPE	Formalin-Fixed and Paraffin-Embedded
FISH	Fluorescent *in situ* hybridisation
GAGs	Glycosaminoglycans
HCC	Hepatocellular carcinoma
HCV	*Hepatitis C virus*
IRES	Internal ribosome entry site
NBF	Neutered buffer formaldehyde
ncp	Noncytopathic
NGS	Next-generation sequencing
NS Protein	Nonstructural Protein
ORF	Open reading frame
PCR	Polynucleotide chain reaction
qRT-PCR	Quantitative RT-PCR
RaPestV-1	*Rhinolophus affinis* Pestivirus
RdRp	RNA dependent RNA polymerase
RNA	Ribo Nucleic acid
RT-PCR	Reverse transcriptase PCR
ss	Single-stranded

UTR	Untranslated regions
YVF	Yellow fever virus

Introduction

To the family of *Flaviviridae* belongs many arthropod-borne viruses, that harbour important pathogens infecting mammals as well as birds, including Classical swine fever virus (CSFV), Bovine viral diarrhoea virus type 1 and 2 (BVDV 1-2), Hepatitis C virus (HCV), Yellow Fever virus (YFV) and Zika virus. The *Pestivirus* is the 4th approved genus out of four of the single-stranded positive RNA viruses within the family Flaviviridae[1].
Pestiviruses are capable of infecting prevalently cloven-hoofed animals as well as bats[2] and rats[3] as revealed by recent studies. They are often characterized by their detrimental effect on the embryo or on the foetus, causing subsequent neurological effect, malformations or even stillbirth. In this event, neurological lesions may result in congenital tremor (CT), also known as myotonia congenita or more commonly as "shaking pigs disease", which is characterized by tremors of the head and limbs of newborn piglets[4].

CT of piglets is characterized by generalized severe shaking, resulting in the subsequent incapability of sucking milk, that may lead to an increase in mortality and undernutrition, which implicates a certain risk for domestic pigs health management. Historically, two types of CT have been investigated, where type A is associated with typical genetically inherited histopathological lesion such as variable hypomyelination of the brain and spinal cord, whereas type B lacks those features[5].

Even though CT in piglets has been described already in early reports of the disease by Kinsley (1922) in the United States, only 40 years ago Patterson et al. (1973) suggested that CT could have been linked to an infectious agent. During the last two decades, thanks to recent advances in diagnostics for viral infections, many new viruses have been discovered, like the atypical porcine pestivirus (APPV). APPV was first identified in the USA in porcine serum via metagenomic sequencing[4]. Soon thereafter, the first report regarding reproduction of CT in an animal model was published by Arruda et al. (2016), who was finally able to verify Pattersons' assumption, by inducing CT experimentally. They inoculated the virus in pregnant sows, more precisely in their foetal amniotic vesicle, as well as intranasally and intravenously. They observed that the sows were not infected, yet the recently farrowed piglets were showing signs of CT type A. Concerning the type A of CT, 5 subtypes have been enquired, among which type A-II cases have been considered as the dominant one in the presence of APPV[6]. Three other studies published during the same period of time by de Groof et al. (2016), Postel et al. (2016) and Schwarz et al. (2017), together with the important

results obtained in the experiment by Arruda et al. (2016), suggested the vertical infection route of APPV genome in newborn piglets and it's correlation with CT[5–12].

Although sporadic, APPV is widely distributed and has been reported in North[5] and South[12] America, very recently in China[13,14] and in several European Countries such as Austria[15], Germany[9,16], Sweden[17], the Netherlands[6], Hungary[18] and likely occurs globally.

Literature Review

The very first virus was discovered in 1901 by Walter Reed when he postulated and confirmed the theory that yellow fever could be experimentally transmitted via the life of a particular mosquito species, and that this could be spread to humans by the bite of an infected female mosquito. The yellow fever virus (YFV) is nowadays one of the most representative virus of the family of *Flaviviridae*[1], being just one out of more than 5000 virus species that have been described, although the results of a new study suggest that at least 320,000 different viruses are able to infect mammals[19]. Viruses are found in almost every ecosystem on Earth and are the most abundant type of biological entity[20].

1.0 Viral Taxonomy

1.1 The Flaviviridae family

The *Flaviviridae* family consists primarily of more than 50 species of arthropod-borne viruses, with distinct groups infecting mosquitoes or ticks, mostly having mammals and birds as primary hosts. It includes four different genera: *Flavivirus*, *Hepacivirus*, *Pegivirus,* and *Pestivirus* (Figure 1). Many of the Flaviviruses are host specific and highly pathogenic, such as *hepatitis C* concerning the genus of *Hepacivirus*. A high majority of a member belonging to the *Flaviviridae* family are dangerous human and animal pathogens, e.g. yellow fever virus and dengue virus, Zika virus, *Japanese encephalitis virus*, *West Nile virus* and *Tick-borne encephalitis virus*. The infectious range of the primary host can variate from asymptomatic to severe or fatal haemorrhagic fever, neurological disorder, abortion or fatal mucosal disease[21].

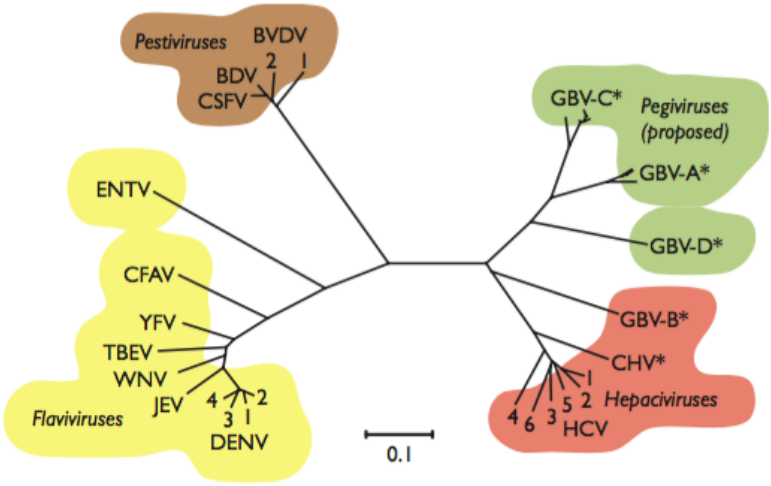

FIGURE 1: GENETIC TREE OF THE *FLAVIVIRIDAE* FAMILY, REPRESENTING INTERRELATIONSHIPS BETWEEN THE FAMILY[1].

1.2 The genera of the Flaviviridae family

1.2.1 Hepacivirus

Current worldwide estimation suggests that over 170 million people have been infected with Hepaciviruses[22]. This genus includes the *Hepatitis C Virus* (HCV) as the best-characterised member[23], a pathogen occurring mainly in humans. Infections are typically persistent and target the liver, causing chronic liver disease, that could lead eventually to cirrhosis and may increase the risk of developing hepatocellular carcinoma (HCC). Other than the HCV, several other viruses of unknown pathogenicity that infect horses, rodents, bats, cows, and primates are included. Among these, the *Non-primate hepacivirus* is affecting dog or horse respectively with *Canine-* or *Equine hepacivirus* causing persistent liver damage[23]. *Hepacivirus* genus is characterized by the common morphology and typical biological properties of the *Flaviviridae* family: their genome is a linear, positive sense, single-stranded RNA molecule of around 9.6 kb. It encodes a single polyprotein that is proteolytically processed by a combination of cellular and viral proteases into structural and nonstructural proteins such as serine protease, RNA helicase, and RNA dependent RNA polymerase (RdRp)[23]. Their limited ability to be propagated in cell culture is the only distinguishing feature compared to the genera *Flavivirus* and *Pestivirus*. The route of transmission is mainly hematogenous due to exposure to contaminated blood caused by improperly sterilized medical equipment and intravenous drug use. Through phylogenetic analysis 6 genotypes were identified. Three (1a, 1b, and 3c) out of these HCV genotypes are spread worldwide due to exposure to contaminated blood, caused by improperly sterilized medical equipment and intravenous drug use[24].

1.2.2 Flavivirus

The *Flavivirus* genus consists primarily of more than 50 species of arthropod-borne viruses. Those include the *Tick borne encephalitis virus* (affecting human and goat in Europe and Asia), *Louping ill* (sheep meningoencephalitis-Europe), *Yellow fever* (humans – tropics area), *Dengue fever* (human – Asia and Africa), *Zika* virus (human – Africa, Asia, Americas), *Japanese encephalitis* (human, swine horse, cattle – Far East), *Murray Valley encephalitis* (sheep, human – Australia), *St. Louis encephalitis* (horse, human – USA), *West Nile virus* (human, horse, bird – Worldwide), *Usutu virus* (wild bird – Europe, Africa), *Israeli turkey encephalomyelitis virus* (turkey – Israel, South Africa) and *Wesselsbron disease* (sheep abortion – Asia, Africa) and several other viruses which may cause mainly encephalitis. Other members cause economically important diseases in domestic or wild animals.

Additional viruses infecting only arthropods or only mammals (e.g., *Tamana bat virus*) have been described recently[21].

Most of these viruses are transmitted by the bite of an infected arthropod (mosquito or tick) and hence, classified as arboviruses[2,23,25,26].

Concerning the host of this genus, birds and mammals are the primary hosts showing a vast range of infections, from asymptomatic to severe or fatal haemorrhagic fever or neurological disease[27].

Other members cause economically important diseases in domestic or wild animals. Additional viruses infecting only arthropods or only mammals (*e.g.,* Tamana bat virus) have been described recently[23].

An importan feature that makes the *Flavivirus* unique compared the *Flaviviridae*, is the presence of a type 1 cap (m^7GpppAmp) at the 5' end of the genome, which is not seen in viruses of other genera[23].

1.2.3 Pegivirus

The *Pegivirus* is the 3rd approved genus of ss positive RNA viruses in the family of *Flaviviridae*. So far, there are eleven named species within the genus. *Pegiviruses* are widely distributed in a range of mammalian species such as horse, bat, chimpanzee, rodent and the human population worldwide, in which they cause persistent infections[1].

In the early 1990's there was still a conspicuous number of a patient affected with hepatitis, that could not be linked to *Hepatitis A virus*. Further research aimed to identify supplementary hepatitis agents, divulged that three novel viruses have been discovered classified as the *Pegivirus* genus based on sequence relatedness and overall genome structure[1], The phylogenetic analysis of Pegiviruses shows, that they are distantly related to other members of the family *Flaviviridae*, forming a distinct cluster. Moreover, they show several differences in genome organization from members of the *Hepacivirus* and other *Flaviviridae* genera such as the possession of an internal ribosome entry site (IRES) element and the fact that they do not encode a protein homologous to the nucleocapsid proteins, feature found in members of other genera in the *Flaviviridae*[28–30].

1.2.4 Pestivirus

It is known that pestiviruses may induce various clinical symptoms depending on age and immune status, as well as on virus species and strain.

Regarding the genus of interest, the genus *Pestivirus* includes some relevant pathogens infecting mainly cloven-hoofed animals, until the recent discovery of several distantly related *Pestivirus* RNA sequences, belonging to a virus able to infect bats and rats. This genus consists of 4 approved species: classical swine fever virus (CSFV), bovine viral diarrhea virus 1 and 2 (BVDV-1, BVDV-2), and border disease virus (BDV)[31], causing remarkable economic damage in livestock industries.

Infections with most pestiviruses yields, besides mild or subclinical disease in the immunocompetent host, acute hemorrhagic disease, as documented for CSFV or highly virulent strains of BVDV-2[32,33].

Pestivirus infections are often characterized by the detrimental effect on the embryo or fetus, causing stillbirth, neurological defects or malformations[34]. On a neurological-based level, dysmyelination or hypomyelinogenesis is a typical lesion in ovine fetuses infected with BDV in the late pregnancy[35].

Historically, CSFV was the only pestivirus known to cause natural infections with clinical significance in swine[36] and usually resulting in different clinical symptoms, like congenital tremor (CT) in affected piglets, with high morbidity and mortality.

During the last twenty years of researches, new kind of viruses was discovered, including the giraffe pestivirus, antelope pestivirus, Hobi virus, Bungowannah virus, Norwegian rat pestivirus and a putative Pestivirus (RaPestV-1) in *Rhinolophus affinis* bats[9]. More precisely in 2003, a Bungowannah virus was isolated from samples of a farm pig in Australia, showing an increase in sudden death in three- to four-week-old pigs and an increase of foetal death at 20-28 weeks of pregnancy[37], but no link to CT was given. After necroscopical examination, multifocal non-supportive myocarditis with myonecrosis was observed[5].

1.2.4.1 Genomic Morphology of Pestiviruses

Pestiviruses are characterized by a small enveloped (50 nm), 40–60 nm in diameter virions with a single core protein and 2 or 3 virus-encoded glycoproteins, and linear, positive-sense single-stranded RNA (ssRNA) genome of approximately 9.0 to 13 kbp, as can be seen in a

diagrammatic representation of the *Pestivirus* genome in figure 2. All members of the genus lack a 3'-terminal poly (A) tract. Replication and assembly of the virions are taking place in the cytoplasm, in the membrane vesicles derived from the endoplasmic reticulum (ER). The assembled virion exits the host cell by budding into the lumen of the ER and is secreted through the vesicle transport pathway. The translation is taking place directly from the genomic RNA, containing a type 1 cap. Humans and mammals serve often as natural "dead-end" host, whereas transmission of the virus is taking place via a vector as ticks and mosquitoes[31].

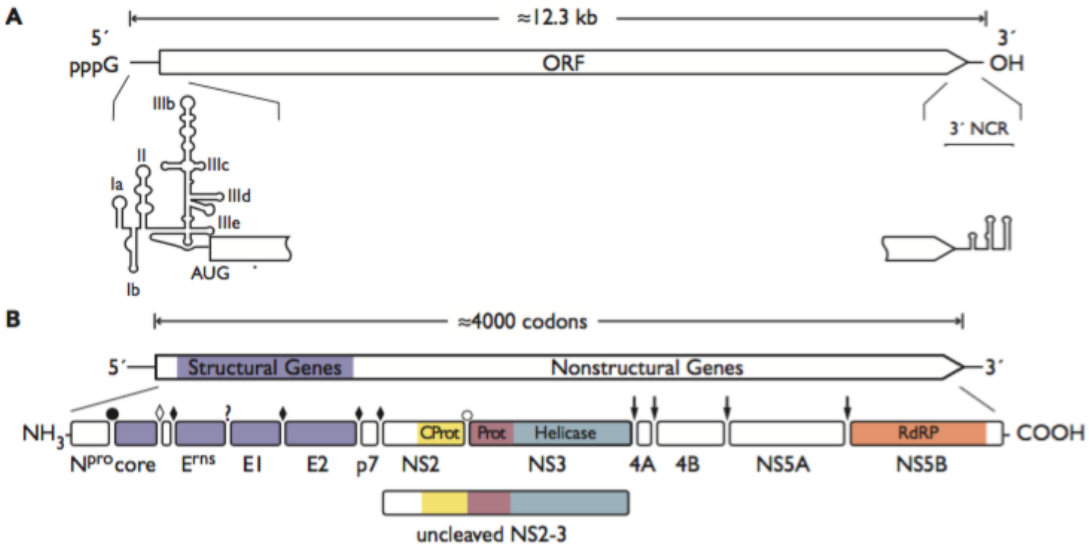

FIGURE 2: PESTIVIRUS GENOME STRUCTURE AND PROTEIN EXPRESSION. A: GENOME STRUCTURE AND RNA ELEMENTS. B: POLYPROTEIN PROCESSING AND CLEAVAGE PRODUCTS[1].

1.2.4.2 Features of the Nonstructural Proteins

Characters such as virion morphology, genome organization, a method of replication, and the number and size of structural and nonstructural viral proteins are used for distinguishing different virus families and genera.

Pestiviruses are classified into two biotypes based on their ability to induce a cytopathic effect in cell culture: noncytopathic (ncp) and cytopathic (cp). Furthermore, the ss +RNA genome of pestiviruses is comprised of a single open reading frame (ORF), which is flanked by 5′ and 3′ untranslated regions (UTR). Translation results in the generation of a single polyprotein consisting of about 3,900 amino acids (aa). The sequential order of viral proteins

in the polyprotein is, among various protein, the NS2–3. The downstream NS proteins are processed by viral Nonstructural (NS) proteases NS2 and NS3–4A[5,38].

Structural proteins are responsible for the structure of the virus, like the capsid, whereas NS proteins are those proteins encoded by a viral genome that are produced in the organisms they infect, but not packaged into the virus particles. Some of these proteins may play roles within the infected cell during virus replication or act in regulation of virus replication or assembly. Nonstructural proteins are proteins that can be either proteases or polymerases, depending on the type of NS proteins[39]. So far, 80% of the APPV's protein profile resembles the one belonging to the *Flaviviridae* family, especially to the genus *Pestivirus*, due to the presence of NS proteins, like the NS2 and the NS3, which were identified in the genome of APPV[5,38].

Uncleaved NS2-3 represents an essential factor for virion morphogenesis, while NS3 is an essential component of the viral replicase. NS2 is a cysteine autoprotease responsible for cleavage of NS2 to NS3, whereas NS3 is a chymotrypsin-like that has serine protease, helicase, and NTPase activity, catalyzing both cis- and trans-cleavage. The APPV NS2 and NS3 proteins shared relatively high identity to the NS2 and NS3 of *Rhinolophus affinis* Pestivirus RaPestV (respectively 60% and 74% identity)[5].

1.2.4.3 Transmission of APPV

In the case of APPV, even though there is not yet a complete picture of how the virus is carried, vertical transmission in sow is the main form of infection. As suggested by the result of the experiments performed by a research group of the Netherlands, three gilts were inoculated via intramuscular injection at day 32 of pregnancy. In two of the three litters, vertical transmission of the virus occurred. More specifically, the infection should have occurred during gestation and not during farrowing, suggesting a trans-placental infection. The thesis of the research group was supported by the fact that none of the sow that farrowed the APPV positive piglets showing CT contained APPV in the serum at the time of delivery, concluding that trans-placental transmission of APPV and subsequent infection of the fetuses is a very likely cause of congenital tremor type A-II in piglets[7,40].

A study of farm piglets born with CT showed that the high viremia in serum declines at five months of age, but the shedding of the virus in feces continues, which explains why the virus remains present at affected farms, causing new outbreaks. Another evidence in support of

the thesis that the virus can also be transmitted via orofecal route is that there is a significant level of APPV genome present in salivary glands, duodenum, pancreas, and colon of the infected sows. Further studies should focus on virus isolation aimed at elucidating the mechanisms underlying transmission and pathogenesis[6].

2.0 Pathogenesis of APPV

Flaviviruses enter the cells via clathrin-mediated endocytosis, which is mediated by the envelope proteins and starts with the attachment of the virion to the cell. Through this process, possibly various attachment factors are increasing the duration of contact, in order to enhance the efficiency of infection. Presumably, several cellular factors could function as attachment factors or receptors during the flavivirus entry, for instance, the binding of flaviviruses through their E proteins to glycosaminoglycans (GAGs) and cellular lectins are well documented. After the successful endocytosis, the virions are transported into late Rab7-positive endosomal compartments, where viral fusion occurs, which is triggered by the acidic endosomal environment. Viral RNA replication begins shortly thereafter in association with membranes of the host cell. The peculiar membrane rearrangements of cells actively replicating the virus particles thought to coordinate the processes of genomic RNA replication and virus assembly. After assembly, virus particles bud into the endoplasmic reticulum and are secreted from the cell[1].

Piglets infected in utero at 45-62 days of gestation are showing CT as a common phenomenon which is characterized by tremors of the head and limbs that can vary in severity[7]. The tremors last for weeks to months, but most piglets are clinically normal at weaning. It has long been assumed that Type A-II CT is caused by APPV, as the disease can be replicated in piglets though experimental infection of sows with homogenates of the cerebellum, cerebrum and spinal cord of clinically affected piglets[10], or by inoculation of sows during early pregnancy with serum containing high levels of APPV[6,7]. Furthermore, most diseases occur in gilts recently introduced into herds, and it is extremely rare for the same sow to give birth to piglets with CT after a first litter with shaking piglets, suggesting that a degree of immunity develops to the disease. After further research, high APPV RNA load was detected in lymphoid organs, suggesting that these constitute the main target for APPV replication[9].

The general clinical outcome of pestivirus infection is associated with the stage of gestation at which infection occurs. If the infection occurs in an early stage of pregnancy prior to the

establishment of fetal immunocompetence, that would result in persistently infected offspring which are immunotolerant to the virus. Whether this occurs with APPV is unknown, but recent studies have described piglets persistently infected with APPV which do not appear to generate an antibody response to the virus[11].

However, the pathogenesis and etiologies of APPV are currently speculative and further experimental infections of pregnant sows with a virus isolate will be required to finally fully understand the mechanisms of pathogenesis of this novel pestivirus.

3.0 Clinical Signs of APPV

CT is generally classified in two types of disease (types A-I – A-V and type B) according to their presence (type A-I – A-V) or absence (type B) of histopathological lesions, more precisely related to various hypermyelination of spinal cord and brain. Type A is furthermore divided into five categories, depending on the etiological background of the congenital tremor, whereas A-I cases are associated with viral infection caused by *Classical Swine Fever* (CSFV) and characterized by cerebellar hypoplasia[41]. The cause for type A-III is linked to a genetic background affecting only the Landrace breed (sex-linked recessive, where the absence of tight myelin sheaths and fewer oligodendrocytes was observed[41], while the cause for type A-IV is an autosomal recessive genetic defect present only in Saddleback breed defined by cerebrospinal hypomyelinogenesis[4,42,43]. CT of type A-V is related to intoxication that is often associated with organophosphorus-treated food, caused by trichlorfon toxicosis[42] and delineated by cerebellar hypoplasia[42,44]. Type A-II cases have been the most puzzling cases so far[10,45,46] , recent researches have proven that CT type A-II is associated with the atypical porcine pestivirus (APPV)[6,7,9,11,14].

CT is best described as newborn piglets showing local or systemic muscular spasm, ataxia and inability to stand and suck, observed within hours of birth. Typically, the shaking occurs in piglets only when they are awake, as those affected with CT does not tremble during sleeping time. It is not the direct cause of death for the piglets, although the tremor can prevent them to stand and suck properly, resulting in subsequent retarded growth or even death from starvation. Symptoms can be worsened by stress factors, for example, stress and cold, and CT is often complicated with splay legs[4]. The condition decreases as the affected piglets grow, this means that piglets have physiological vital parameters at weaning age, although in some severe cases, tremor persists even into adult age[10].

Aims

In 2017 we investigated CT cases of piglets in a particular farm in Hungary, that's most probably related to APPV infection. The aims of our research are (i) to detect the virus in affected piglets by RT-PCR, (ii) gaining partial genome sequences of the virus (iii) to compare the identified strains to each other and to similar sequences from the GenBank through phylogenetic analysis, that can help in cognition of the origin, spread and genome evolution of the Hungarian strains, (iv) to assess the distribution of the viral genome in various tissue samples of the affected animals with a new RNA *in situ* hybridization method, RNAscope.

Materials and Methods

4.0 Samples collection

For diagnostic purposes, 1–3 day-old piglets originating from 2 farms in Hungary were humanely euthanized according to the current Hungarian animal welfare regulations (Number of the pilot license: PE/EA/544-2/2018). General anesthesia of the piglets was induced with the combination of xylazine and ketamine injected into the ear canal, then T61 was injected behind the shoulder, in the ventral area of the ribs.

Regarding the first farm, all the 4 piglets (Animal A, B, C, D) were experiencing problems with CT and subsequent growth retardation. In the second farm, three piglets were not affected by CT. These cases of CT were brought to our attention by the responsible veterinarians, which could observe several similar cases all over the country. For diagnostic evaluation cerebellum, brainstem, cerebrum, tonsil, kidney, tracheal lymph nodes, spleen, thymus, and mesenteric lymph nodes samples were dissected and collected. Subsequently, one part was fixed in 10% neutrally buffered formaldehyde (NBF) for 24 hours whereas the second part was frozen at −80 ℃ for further analysis.

5.0 RNA extraction

Homogenates (10%) were made from frozen samples in sterile PBS on ice. Homogenization was performed in TissueLyser LT (QIAGEN). This homogenized material was then centrifuged at 3000 × rpm for 3 minutes at RT. We extracted the RNA from 140 µl of supernatant with QIAamp Viral RNA Mini Kit (QIAGEN) by the use of QIAcube (QIAGEN). We eluted the RNA in 60ul of elution buffer.

6.0 Virus detection

6.1 Molecular biological tools for virus detection – the RT-PCR

There are various tools of molecular biology for the detection of viruses. Because of the complicated structures of antigens and the similarity of pestiviruses on the protein level, it is easier and more reliable to use RNA based methods instead of immunological assays.

6.1.1 RT-PCR

Reverse-transcription polymerase chain reaction (RT-PCR) is an established two-step process, which is a simple and effective method for qualitative RNA detection. First RNA is reverse transcribed by the use of random hexamers and reverse transcriptase into its DNA

complement. Subsequently, the newly synthesized DNA (cDNA) containing the reversed transcriptied ssRNA will then be exponentially amplified using a conventional PCR. The PCR will allow reading the results as "positive" (presence of the RNA) or "negative" (absence of the RNA) of the amplified gene product using agarose gel electrophoresis[47].

Reverse transcription was performed using the OneStep RT-PCR kit (QIAGEN) according to Table 1, with primers targeting a 120 bp long sequence of the NS2–NS3 protein coding region[9].

TABLE 1: THE AMOUNT OF REAGENT USED FOR ONE RT-PCR REACTION

Reagent	Volume (µl)
QIAGEN OneStep RT-PCR Enzyme Mix	0.4
QIAGEN OneStep RT-PCR Buffer,* 5x	2.0
dNTP Mix, 10 mM	0.4
RNase-free water	5.7
RNase-inhibitor	0.1
Forward primer (40 µM)	0.2
Reverse primer (40 µM)	0.2
Template	1.5

In order to perform the RT-PCR procedure, we mixed all components together in one tube as shown on Figure 5 and started the thermal cycler program, which was the following: stage 1 took 95 ℃ for 15 minutes, 50 ℃ for 30 minutes; 95 ℃ for 30 seconds, 60 ℃ for 30 seconds, 72 ℃ for 30 seconds in stage 2 (40 cycles); 72 ℃ for 5 minutes as final extension.

6.1.1.1 Sanger sequencing

PCR products were subjected to gel electrophoresis and amplicons with suitable length were cut out of the gel and purified by the Qiagen Gel Extraction Kit (Qiagen). Bidirectional Sanger sequencing reaction was performed with BigDye™Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Ljubljana, Slovenia) with primers targeting a longer segment (1600 bp) of the NS2-3 coding region, and the capillary electrophoresis was carried out by a commercial provider (Hungarian Natural History Museum).

6.1.1.2 Phylogenetic analysis

The obtained sequences were proofread, assembled using E-INS-i method of the online software mafft version 7[48] and aligned against available APPV genomes downloaded from the GenBank that represent the overall diversity of the virus. Maximum-Likelihood (ML) analyses were carried out with RAxML[49] implemented in raxml-GUI v. 1.3[50]. The GTR+G nucleotide substitution model was used for the partitions with ML estimation of base frequencies, and an ML bootstrap analysis with 1000 replicates was used to test the support for the branches. The phylogenetic trees were visualized and edited using MEGA7[51]. The phylogenetic tree was also inferred using the Bayesian method implemented in MrBayes v3.1.2[52], using the same substitution model. Four Markov chains were run for 10 000 000 generations and sampled every 1 000 generations with a burn-in value set at 6 000 sampled trees.

6.2 Histological tools for virus detection – RNAscope

A novel method of *in situ* hybridization was used to visualize ss RNA molecules in samples mounted on slides. RNAscope main advantage is the non requirement of the RNA-free environment used for traditional ISH. The assays are based on Advanced Cell Diagnostics' (ACD) patented signal amplification and background suppression technology.

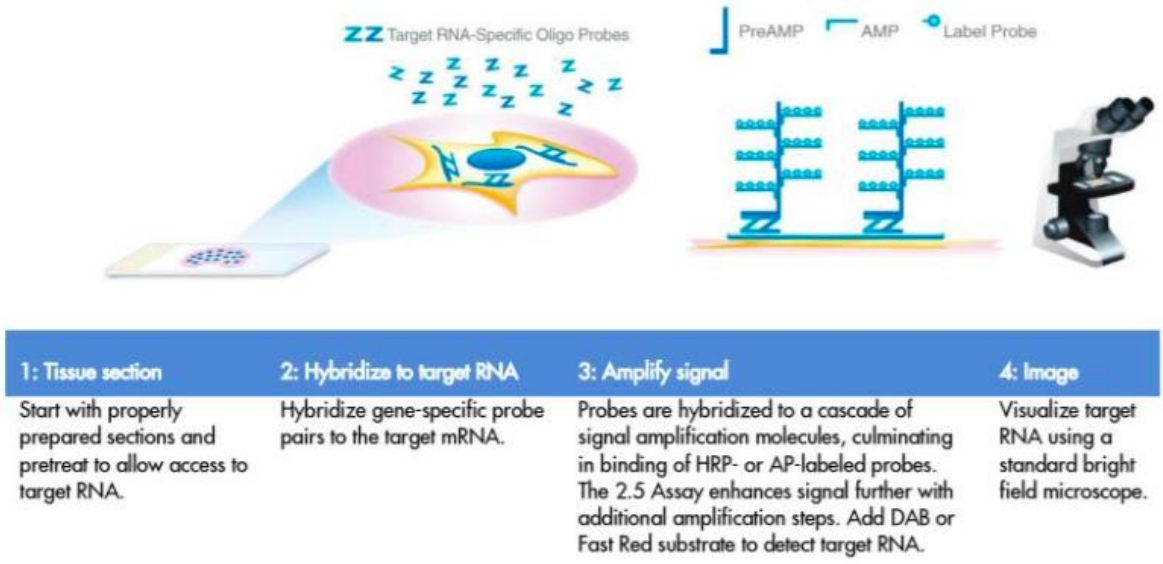

FIGURE 3: SCHEMATIC REPRESENTATION OF THE RNASCOPE ASSAY PROCEDURE. STEP 1: SPECIMENS ARE FIXED AND PERMEABILIZED FOR TARGET PROBE ACCESS. STEP 2: TARGET RNA-SPECIFIC OLIGONUCLEOTIDE PROBES (Z) ARE HYBRIDIZED IN DOUBLE Z PROBE (ZZ) TO MULTIPLE RNA TARGETS. STEP 3: MULTIPLE SIG SIGNAL AMPLIFICATION MOLECULES ARE HYBRIDIZED, EACH RECOGNIZING A SPECIFIC TARGET PROBE, AND EACH UNIQUE LABEL PROBE IS CONJUGATED TO A DIFFERENT ENZYME. STEP 4: SIGNALS ARE DETECTED USING A STANDARD BRIGHT-FIELD MICROSCOPE (FOR ENZYME LABEL)[53].

The schematic RNAscope assay is illustrated in Figure 3. The procedure can be divided and completed over two days. Tthe RNAscope assay reagents are available in ready-to-use dropper bottles and provide a simple workflow, nearly without the need to pipette. The first step involves properly prepared tissue samples. The sections are first pre-treated, and subsequently, RNA-specific probes are hybridized to target RNA. The signal is amplified using a multi-step process, followed by hybridization to alkaline phosphatase (AP)-labelled probes. Preamplifier, amplifier, and AP-labeled oligos were then hybridized sequentially, followed by chromogenic precipitate development in order to be detected. The single RNA transcript is finally visible using a common bright field microscope at 400–600× magnification, and appear to be a distinct dot. The RNAscope 2.5 assay allows us to observe the results under 100–200× magnification after additional amplification steps, thanks to a red detection kit (Fast Red), which enable RNA molecules to be visualized as red dots[53].

6.2.1 Sample preparation for RNAscope

The procedure was performed manually using RNAscope 2.5 HD Detection Kit (RED) (Advanced Cell Diagnostics, Inc.) and RNAscope Probe specific to the sequence region spanning nucleotides 1–2816 of the genes encoding the NS2 and NS3 proteins, according to the manufacturer's instructions. 5–10 µm formalin fixed, paraffin embedded (FFPE), freshly segmented organ samples were made, then pretreated with heat and protease prior to hybridization with the target oligo probes. The sections were heat treated for an hour at 60 ℃, washed in xylene 2 × 5 minutes, incubated in 100% ethanol for 2x1 minutes and finally air-dried for 5 minutes. We added 2–8 drops of hydrogen peroxide at each section for 10 minutes at room temperature (RT, 20 ℃), we washed them in distilled water 2 times. We placed the slides into boiling 1× Target Retrieval solution for 15 minutes and then immediately washed it in distilled water and repeated in fresh distilled water. Finally, we washed the slides in fresh ethanol, air-dried and drew around the sections with a barrier pen (ImmEdge hydrophobic barrier pen, ACD biotechne). We let it dry overnight at room temperature (RT). The following day, we set the HybEZ oven to 40 ℃ and warm HybEZ Humidity Control Tray containing wet Humidifying Paper for 30 minutes, then we add 5 drops of Protease Plus reagent to each section, placed it into the warm tray and inserted back the sealed tray into the HybEZ oven for 30 minutes at 40 ℃. Subsequently, we washed the slides in fresh distilled water 2 times.

6.2.2 *RNA in situ hybridization*

We warmed the probes at 40 ℃ for 10 minutes and let it cool down to RT. Thereafter, we applied four drops of the proper probe to each slide. The samples were quality controlled for RNA integrity with an RNAscope probe specific to Ss-PPIB gene and for the background with a probe specific to bacterial dapB RNA. We incubated the probes with sections for two hours at 40 ℃, then washed them each two times with 1× Wash Buffer for two minutes. After performing each amplification step we washed the slides with wash buffer every two times for two minutes. The RNAscope workflow is schematized in figure 4.

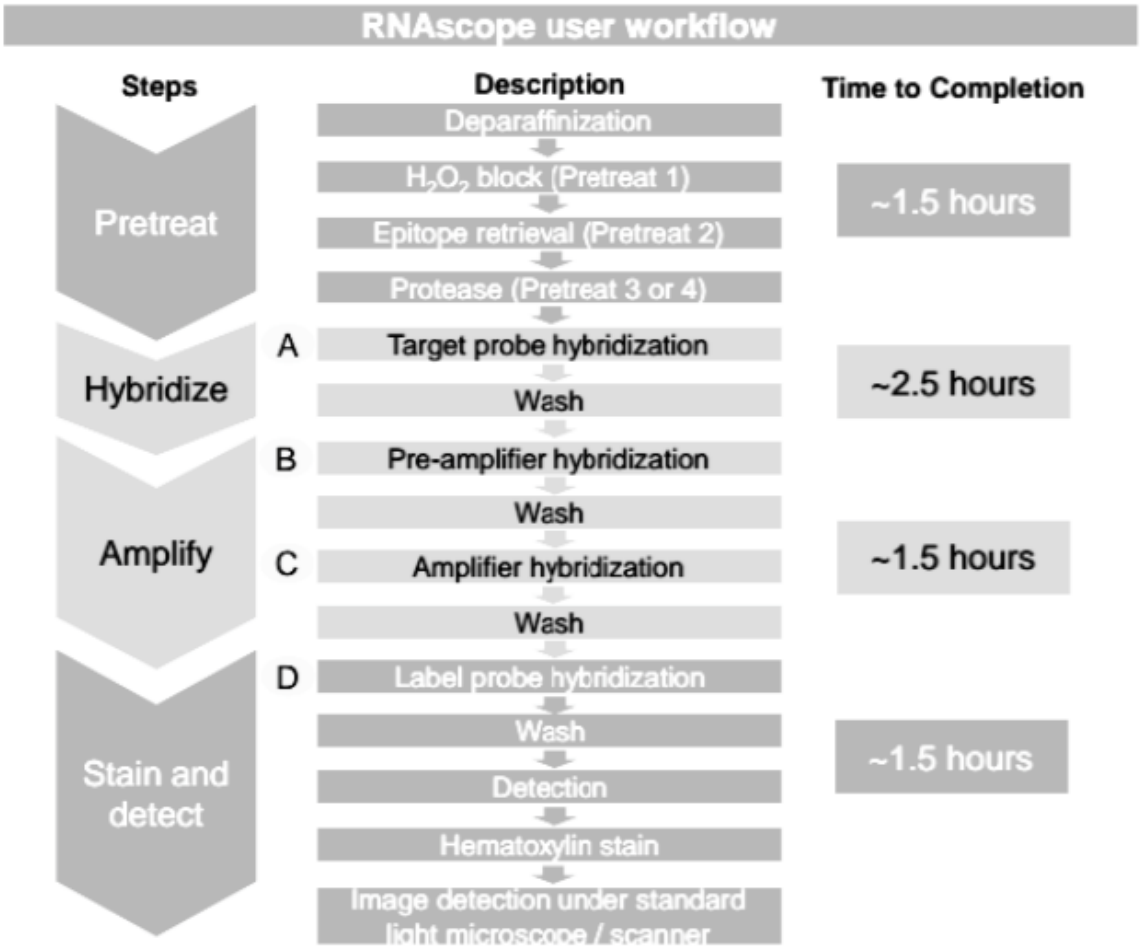

FIGURE 4: A SIMPLIFIED AND SCHEMATIC OVERVIEW OF THE RNASCOPE WORKFLOW STEP-BY-STEP[53].

The Amplification Assay was running in the order described below.

1. Hybridization Amp 1: Apply 4 drops, incubate 30 minutes at 40 ℃
2. Hybridization Amp 2: Apply 4 drops, incubate 15 minutes at 40 ℃
3. Hybridization Amp 3: Apply 4 drops, incubate 30 minutes at 40 ℃
4. Hybridization Amp 4: Apply 4 drops, incubate 15 minutes at 40 ℃
5. Hybridization Amp 5: Apply 4 drops, incubate 30 minutes at RT
6. Hybridization Amp 6: Apply 4 drops, incubate 15 minutes at RT

6.2.3 Detection of the signal

We mixed one volume of RED-B to 60 volumes of RED-A within 5 minutes before use. We applied about 120 µl of RED solution onto each tissue section and incubated it for 10 minutes at RT in the sealed tray. After that, we washed the slide for 2 times with fresh distilled water, placed the slides in Hematoxylin II for 1 minute and then washed it in fresh distilled water 2 times. We submerged the sections in 0.02% Ammonia water for 10 seconds and then washed in fresh distilled water.

6.2.4 Mounting of the slides

We placed the slides in a dry oven for at least 15 minutes at 60 ℃ until they dried completely. We dipped the slides into fresh xylene and immediately placed 1-2 drops of EcoMount. We placed a coverslip over each section. Finally, we let them dry for 5 minutes at RT. Afterward, we analyzed the results by light microscope and specific RNA staining signal was identified as red, punctate dots.

Results

7.0 Morphological Results based on the RT-PCR method

Below we will show our diagnostic results concerning the histological and molecular tests that we performed on the piglets. First, we analyzed them by RT-PCR and then proceed with the *in situ* RNA hybridization method. This allowed us to show sample-by-sample images of the results.

A total number of 9 samples were dissected from seven 1–3 day-old piglets originating from 2 farms in Hungary. Regarding the first farm, all the 4 piglets (Animal A, B, C, D) were experiencing cases of CT becoming evident by unintended shivering. In the second farm, the three piglets were not affected by CT. The piglets were sacrificed for pathological and virological investigations. After beeing dissected, we isolated RNA from the frozen samples and detected the virus by conventional RT-PCR. The assay identified APPV genomes in all of the four clinically affected piglets that showed APPV genomes in cerebellum, brainstem cerebrum tonsil, kidney, thoracic lymph nodes and spleen, suggesting the presence of APPV in all our samples except in the thymus and mesenteric lymph nodes of animal A. All the three piglets without tremor were tested negative.

Reverse transcription was performed using the OneStep RT-PCR kit (QIAGEN) and the semiquantitative results were as follow according to Table 2.

TABLE 2: SEMIQUANTITATIVE RESULTS OF RT-PCR ON ANIMAL A, B, C, D AND *IN SITU* RNASCOPE RESULTS OF ANIMAL D.

Tissue Samples	**RT-PCR**					***In situ* RNAscope**
	Animal	A	B	C	D	D
1. Cerebellum (D1)		+	+	+	+	+
2. Brain stem (D2)		+	+	+	+	+
3. Cerebrum (D3)		+	+	+	+	+
4. Tonsil (D4)		+	+	+	+	+
5. Kidney (D5)		+	+	+	+/-	+
6. Thoracic Lymph nodes (D6)		+	+/-	+	+/-	+
7. Spleen (D7)		+	+/-	+	+	+
8. Thymus (D8)		-	+	+	+/-	+
9. Mesenteric Lymph nodes (D9)		-	+	+	+	+

Concerning piglet A, B, C, and D showing CT symptoms, the results were showing that regarding animal A, the presence of APPV (+) in all our samples except in the thymus and mesenteric lymph nodes could be detected. In animal B, all samples were detected positive for APPV as shown in Figure 5, even though the samples B6 and B7 (respectively the thoracic lymph nodes and the spleen sample) were paler compared to the rest of the samples, suggesting the presence of a smaller amount of RNA in these organs (+/-). Animal C was considered to be full positive, according to the presence of RNA in all the nine samples analyzed. Finally, animal D was also considered to be positive, but, like in animal B, it was possible to see a paler sign on the agarose gel, meaning that it was possible to detect only a smaller amount of RNA in sample D5, D6 and D9 (+/-), respectively in the kidney, thoracic and mesenteric lymph nodes.

As regards the three piglets from the second farm that were not affected by CT, no APPV was present in any of the nine samples dissected.

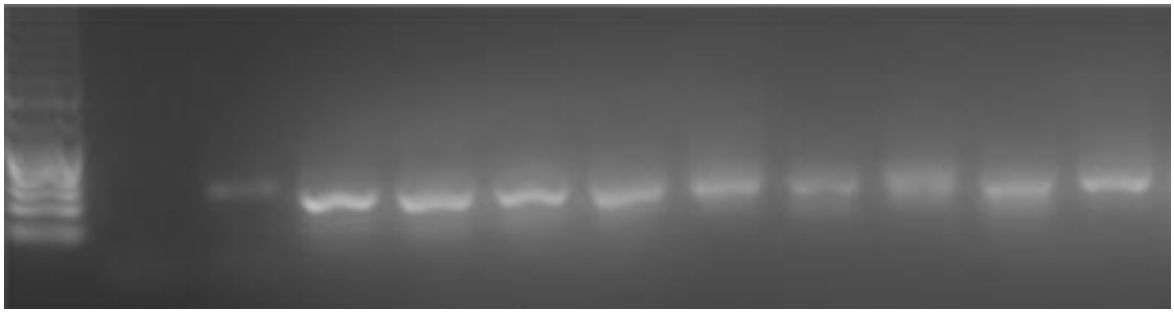

FIGURE 5: QUALITATIVE RT-PCR ELECTROPHORETOGRAM OF ANIMAL B SHOWING THE PRESENCE OF APPV IN VARIOUS TISSUE SAMPLES. FROM LEFT TO RIGHT: 50 BP DNA LADDER, NEGATIVE CONTROL, POSITIVE CONTROL, CEREBELLUM, BRAIN STEM, CEREBRUM, TONSIL, KIDNEY, TRACHEAL LYMPH NODES, SPLEEN, THYMUS, AND MESENTERIC LYMPH NODES SAMPLES. THE RESULTS SHOW THE FOLLOWING: ALL SAMPLES ARE MARKED AS POSITIVE (+).

8.0 Histological Results based on in situ RNAscope hybridization method

In relation to the *in situ* RNAscope hybridisation method, the procedure was performed manually using an RNAscope Probe specific to the sequence region spanning nucleotides 1-2816 of the genes encoding the NS2-3 proteins only on animal D. The results were as follow: RNA was detected as positive (+) in the cerebellum, brainstem, cerebrum, tonsil, kidney, tracheal lymph nodes and in the spleen, and it had a weak signal in the thymus and in the mesenteric lymph nodes samples. The results were summarized in Table 2 and are shown

sample-by-sample in the images below displaying our results. The validation was done in comparison to a positive and a negative control, which we have performed with every assay.

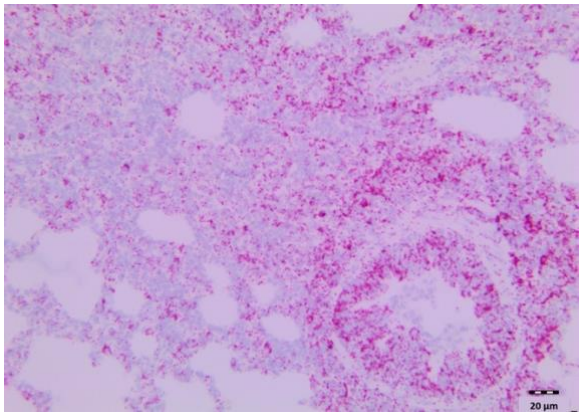

FIGURE 6: POSITIVE CONTROL ASSAY ON LUNG TISSUE. WE USED THE SPECIES-SPECIFIC *SUS SCROFA* PEPTIDYLPROLYL ISOMERAS B (SS-PPIB) PROBE (*IN SITU* HYBRIDIZATION, 200×; BAR = 20 µM)

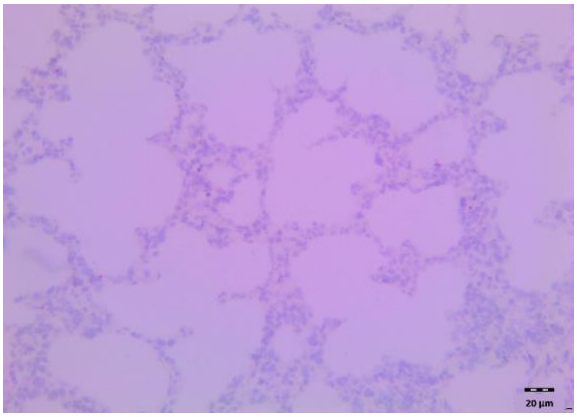

FIGURE 7: NEGATIVE CONTROL ASSAY ON LUNG TISSUE. WE USED THE BACTERIAL DIHYDRODIPICOLINATE REDUCTASE (DAPB) PROBE (*IN SITU* HYBRIDIZATION, 200×; BAR=20µM

Figure 6 and 7 show respectively the positive and negative control assay result we performed on a random swine tissue (here: lung). The samples were quality controlled for RNA integrity with an RNAscope probe specific to *Sus scrofa* peptidylprolyl isomerase B (Ss-PPIB) gene and for the background with a probe specific to bacterial dihydrodipicolinate reductase (dapB) RNA. On the positive control, if the assay is carried out properly, red dots in the cytoplasm of the lung cells should be seen, whereas no coloration should be observed in the nucleus, as in figure 7. On the other hand, the negative control expressed in bacterial cells should be free of red dots. The background should result blue as seen in figure 8, due to the haematoxylin stain.

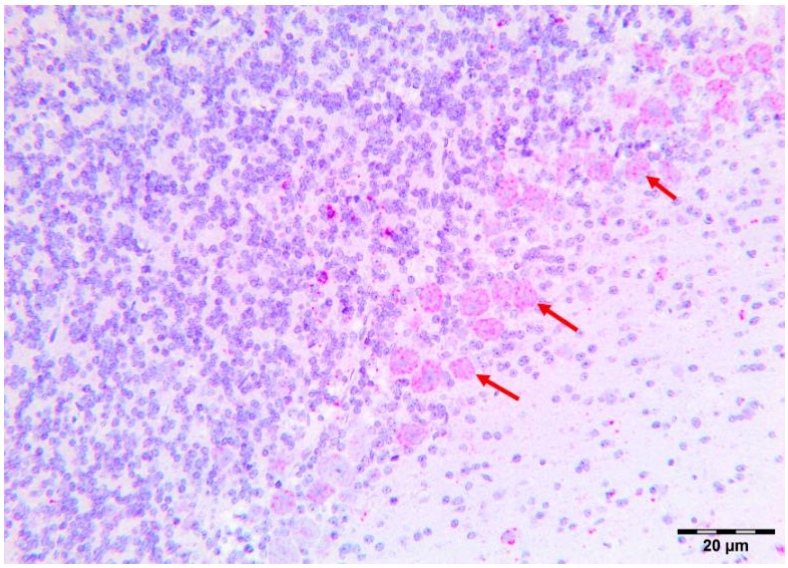

FIGURE 8: SAMPLE D1 – RED COLOUR INDICATES THE APPV VIRAL GENOME IN THE INNER GRANULAR CELLS AND PURKINJE CELLS (RED ARROWS) OF THE CEREBELLUM (*IN SITU* HYBRIDIZATION, 200×; BAR = 20 µM)

Figure 8 is presenting the result of RNAscope showing the cerebellum infected by atypical porcine pestivirus (APPV) in a one to three-day-old piglet with congenital tremor. RNA can be seen as red dots in the internal cells of the granular layer and in the Purkinje cells.

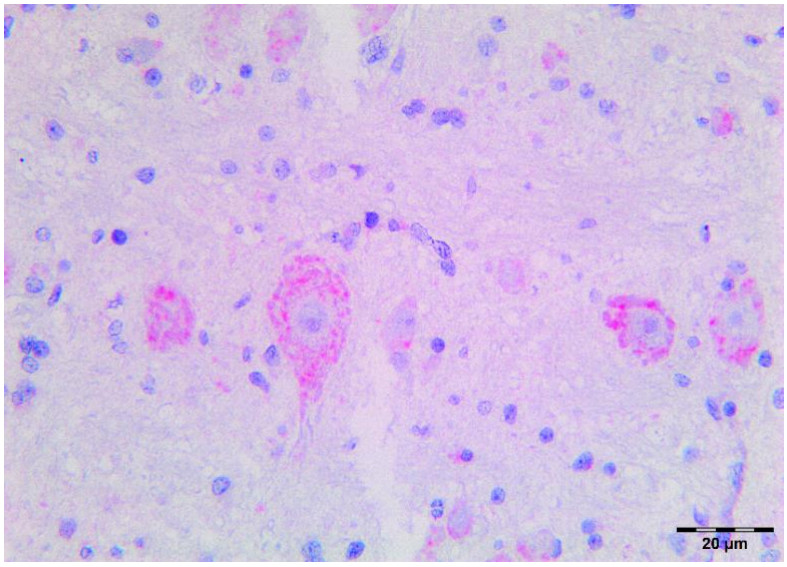

FIGURE 9: SAMPLE D2 – RED COLOUR INDICATES THE APPV VIRAL GENOME IN THE NEURONS OF THE BRAINSTEM (*IN SITU* HYBRIDIZATION, 400×; BAR = 20 µM)

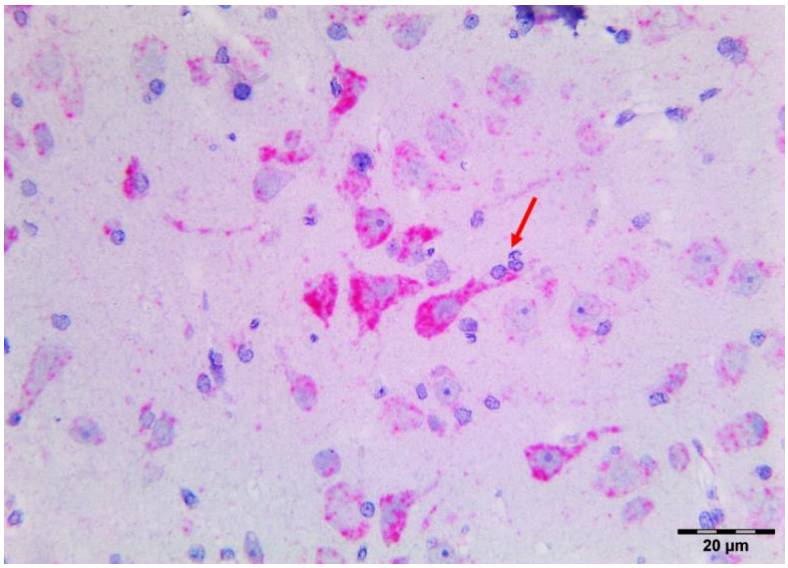

FIGURE 10: SAMPLE D3 – RED COLOUR INDICATES APPV VIRAL GENOME IN THE CORTICAL REGION OF THE CEREBRUM, MICROGLIAL PHAGOCYTIC CELLS OBSERVED NEAR A DYING NEURON (RED ARROW) (*IN SITU* HYBRIDIZATION, 400×; BAR = 20 µM)

RNA positive results can be seen in the brainstem on figure 9 and in the cerebrum on figure 10. Highly colored red dots can be observed in the cortical region of the cerebrum indicating a strong positive result. Moreover, neuronophagia, in which degenerating, dying neurons are surrounded by microglial phagocytic cells, are also observed in the cortical region. Concerning the neurons in the medullar region of the cerebrum, they appear also to be as red dots implying a positive result.

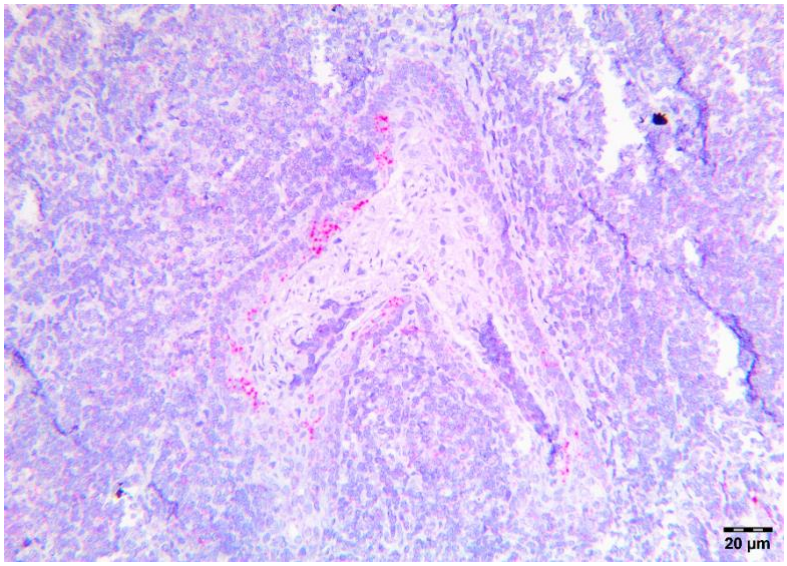

FIGURE 11: SAMPLE D4 – RED COLOR INDICATES THE APPV VIRAL GENOME IN A TONSILLAR CRYPT *IN SITU* HYBRIDIZATION, 200×; BAR = 20 µM)

As shown in Figure 11 positive signals were detected in moderate amounts in the cytoplasm of the epithelial cells of the tonsillar crypts.

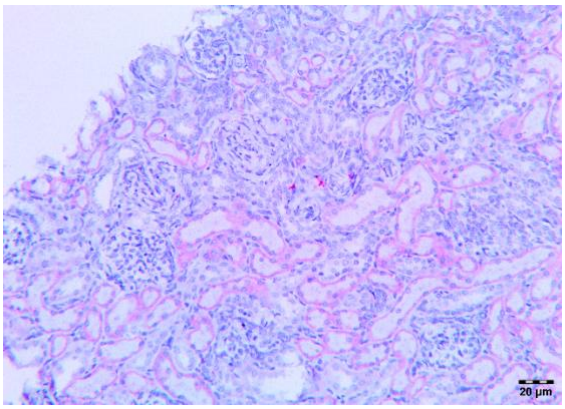

FIGURE 12: SAMPLE D5 – RED COLOUR INDICATES APPV VIRAL GENOME IN KIDNEY CELLS AND LIGHTER RED STAINING INDICATES NONSPECIFIC BINDING IN THE TUBULES (*IN SITU* HYBRIDIZATION, 200×; BAR = 20 µM)

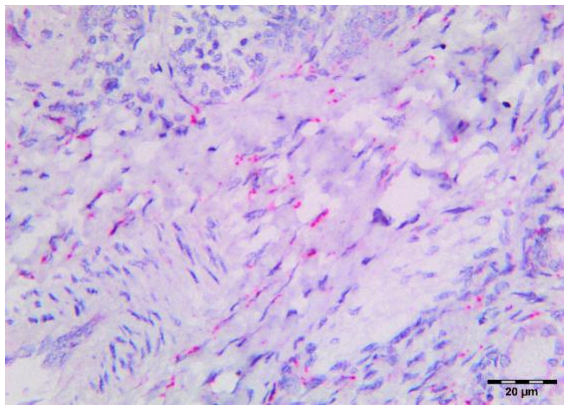

FIGURE 13: SAMPLE D9 - RED COLOUR INDICATES APPV VIRAL GENOME IN THE MESENCHYMAL CELLS OF THE MESENTERIC LYMPH NODE (*IN SITU* HYBRIDIZATION, 400×; BAR = 20 µM)

In figure 12 and 13, a positive APPV affected kidney and mesenteric lymph node tissue sample can be observed. Red dots implying the presence of APPV can be seen in the mesenchymal cells of both samples. Unexpectedly, the virus was only detectable in these cells. Moreover, possible nonspecific binding in the tubules can be detected in figure 11. Finally, positive red dots showing APPV RNA could be detected in the mesenchymal cells of the mesenteric lymph nodes of the affected piglet.

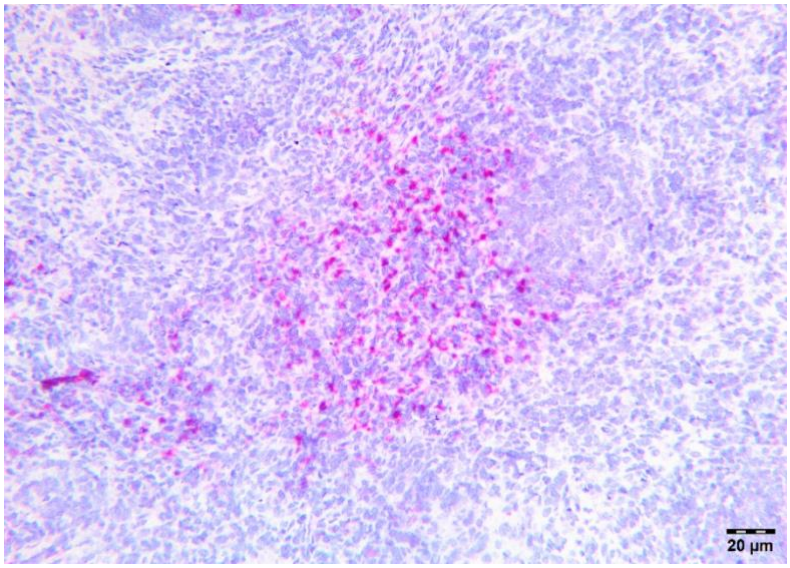

FIGURE 14: SAMPLE D6 - RED COLOUR INDICATES THE APPV VIRAL GENOME IN THE PARA-CORTICAL REGION OF THE TRACHEAL LYMPH NODE (*IN SITU* HYBRIDIZATION, 200×; BAR = 20 µM)

On Figure 14 positive signals are shown in unidentifiable lymphoid-like cells of para-cortical areas of the tracheal lymph node surrounding the lymphoid follicles.

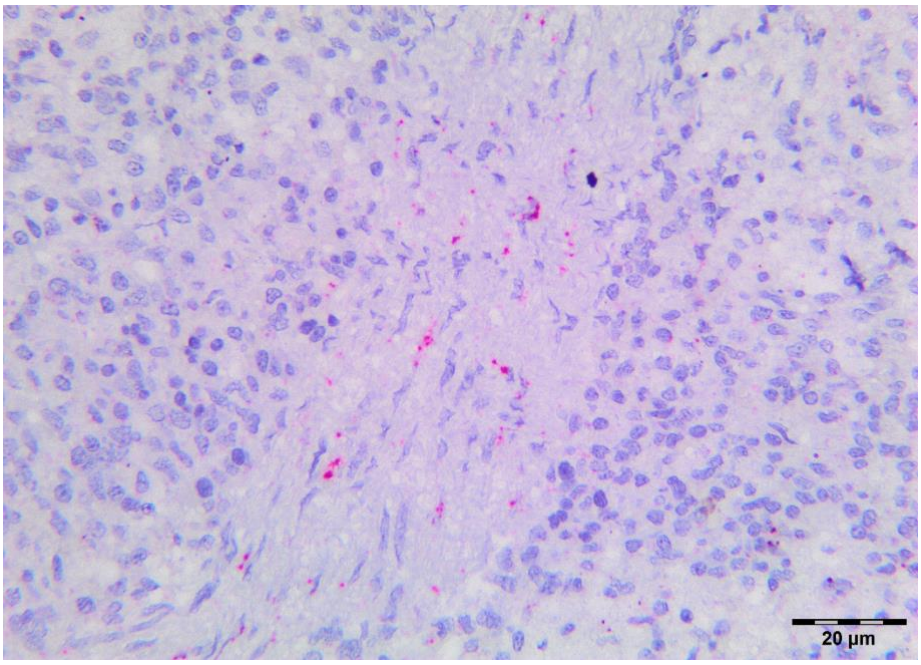

FIGURE 15: SAMPLE D7 – RED COLOUR INDICATES APPV VIRAL GENOME IS DIFFERENTIATED MESENCHYMAL CELLS OF THE SPLENIC TRABECULES (*IN SITU* HYBRIDIZATION, 400×; BAR = 20 µM)

In figure 15 a positive APPV affected splenic tissue sample can be observed. Like in the kidney sample, the red dots can only be detected in the mesenchymal cells, which remains very uncommon.

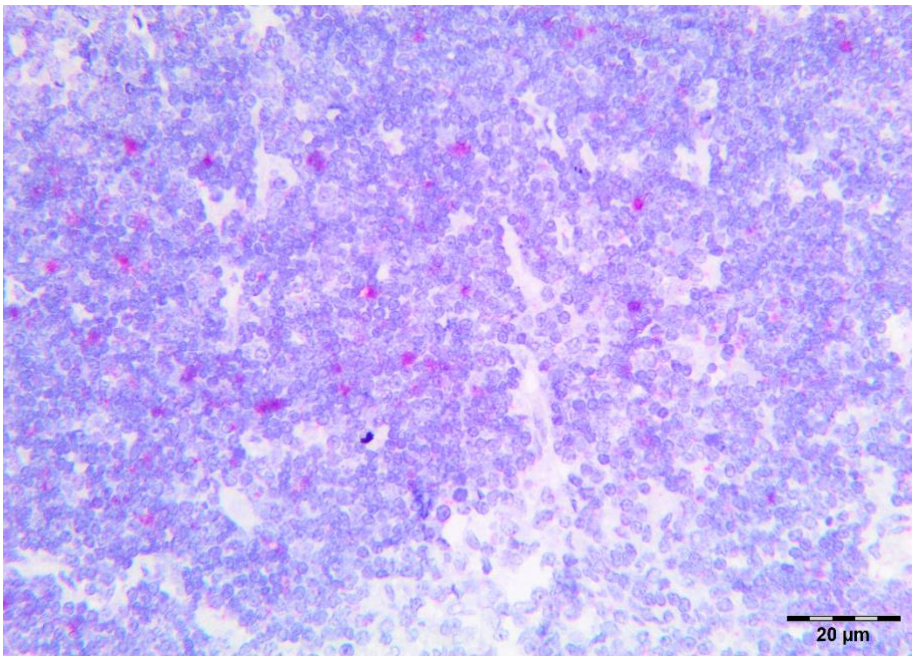

FIGURE 16: SAMPLE D8 – RED COLOUR INDICATES THE APPV VIRAL GENOME IN THE THYMUS (*IN SITU* HYBRIDIZATION, 400×; BAR = 20 µM)

On Figure 16 positive signals are shown in the cortical cells of the thymus.

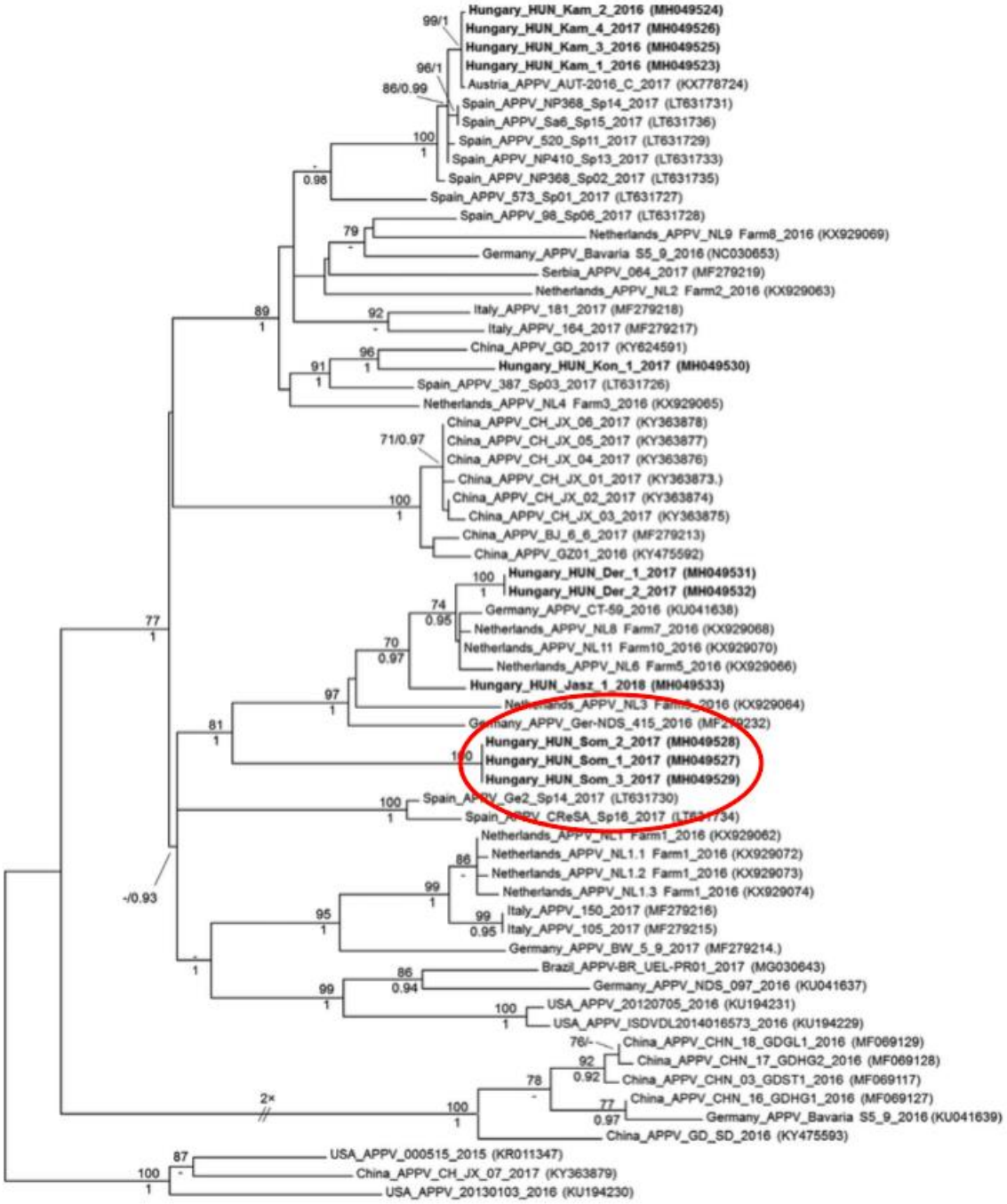

FIGURE 17: PHYLOGENETIC TREE DISPLAYING RELATEDNESS OF THE NS2-3 POLYPROTEIN CODING GENE OF APPV REFERENCE SEQUENCES FROM GENBANK AND HUNGARIAN STRAINS. MAXIMUM LIKELIHOOD BOOTSTRAP SUPPORT VALUES (≥70) ARE SHOWN AS PERCENTAGES BEFORE SLASHES OR ABOVE BRANCHES, BAYESIAN POSTERIOR PROBABILITIES (≥0.90) ARE SHOWN AFTER SLASHES OR BELOW BRANCHES. THE SCALE BAR INDICATES 0.01 EXPECTED CHANGES PER SITE PER BRANCH. STRAINS DISPLAYED ON THE PHYLOGENETIC TREE ARE CODED LIKE COUNTRY OF COLLECTION NAME OF STRAIN COLLECTION DATE (ACCESSION NUMBER)[18].

Recently, our research group investigated the genome of APPV in 25 cases of CT affected piglets from 2005, 2007, 2010 and 2016–2018, originating from six Hungarian farms. They identified APPV in all affected piglets by RT-PCR and sequenced the partial NS2–3 regions. Based on their phylogenetic analysis, Hungarian strains show a high degree of variability

and are clustered into five distinct lineages. Figure 17 is showing the phylogenetic tree displaying the relatedness of the NS2–3 polyprotein coding gene of representative APPV strains from the GenBank and Hungary[18]. The red circled strains are showing the sequences originating from the four piglets from the Hungarian farm, I investigated. We were able to sequence the NS2–3 polyprotein coding region for animal A B D only. The phylogenetic analyses revealed identity among the APPV strains extracted from the three piglets, suggesting that only one APPV strain was responsible for the infection in this particular farm. Furthermore, we could also observe strong similarity to APPV strains originating from Germany and the Netherlands.

Discussion

Viruses of the *Pestivirus* genus are highly variable RNA viruses within the family of *Flaviviridae* causing economically consistent diseases in swine, cattle sheep and goat. In the last 20 years an increasing number of novel pestiviruses were identified in wild ruminants as well as pigs. Among those, the atypical porcine pestivirus has been discovered in 2015 in apparently healthy domesticated pigs in the USA. Subsequent to this finding, different APPV outbreaks were detected in several European countries, in America, and in Asia as well, showing that APPV has probably spread worldwide. Even though an association of APPV with disease remained elusive for many decades, further experimental infectious studies have already demonstrated how pregnant sows inoculated with APPV positive blood or tissue-homogenates are farrowing piglets affected by A-II type congenital tremor (CT). CT is characterized by tremor of the head and limbs of the offspring and is usually complicated by splayed leg and abnormal posture. These symptoms can prevent the piglets to find a teat to suckle, resulting in failing from proper nutrition. Whereas the shaking itself is not the direct cause of death, this unfortunate result of the CT affected piglets will decrease their growth rate and even lead to death by starvation in severe cases.

Here we report the identification and characterization of an atypical porcine pestivirus strain present in Hungary. Recently, many pigs of different Hungarian herds were showing signs of CT, in agreement with the findings of Arruda et al.[7], and therefore they have been investigated for the presence of APPV. The aim of my research was to detect the virus in four 1 to 3-day-old CT affected piglets and further three non-CT affected piglets originating from two different Hungarian swine herds, to characterize the strains and compare them to each other and to previously published sequences that can help in cognition of their origin, spread, and genome evolution. The second objective was to localize and visualize the virus in various tissue samples.

To investigate APPV our research group is conducting genome sequence analysis through RT-PCR and in addition, a novel RNA based *in situ* hybridization method, RNAscope, in order to gather information on the localization of the virus in tissue samples. With the acquired results, we also conducted a phylogenetic analysis, to investigate the genetic development and the origins of the virus[18].

According to our results, after preforming an RT-PCR and a subsequent *in situ* RNAscope analysis on nine different tissue samples (cerebellum, brainstem, cerebrum, tonsil, kidney, tracheal lymph nodes, spleen, thymus, and mesenteric lymph nodes) of each piglet, we could detect the presence of APPV in all of the fresh and FFPE samples from the four CT affected piglet, with a prevalent accumulation in the ependymal and subependymal cells of the cerebrum and in the cerebellum, too. APPV was mostly localized in the neurons of the internal part of the granular cell layer and in the Purkinje cells of the cerebellum, but not in the outer cells of the granular layer as observed in infections with classical swine fever[33]. Infection of the aforementioned cells is the possible cause of the clinical signs[9]. Partly similar results were observed in 2016 by Postel et al., who detected the virus genome also in the internal granular cell layer of the cerebellum by fluorescence *in situ* hybridization (FISH), but not in the Purkinje cells. Subsequently, the study carried by de Groof et al.[6] also identified APPV infected cells in the central nervous system, in accordance with our results.

The selection of the nine tissue samples obtained during the necropsy of the piglets was based on previous studies. The detection of APPV in all of the examined samples with a dominance in the cerebellum and brain stem is confirming the results of Arruda et al.[7], suggesting that the viral infection has a widespread, systemic distribution, whereas the clinical impact (development of CT) depends on the infection of the central nervous system, mainly the cerebellum.

A subset of positive samples was selected for sequencing the NS2 and NS3 gene. Phylogenetic analysis revealed that sequences of the NS2–3 region of the APPV strain detected in Farm 1 are closely related to strains from The Netherlands and Germany, suggesting that the virus has arrived in Hungary through international live pig trade or semen, which is similar to what our research group has found in the case of PRRSV[54].

In summary, piglets showing clinical signs of CT type A-II develop a long-lasting, yet sometimes resilient infection with APPV. The mechanism of the central nervous system dysfunction in the clinically infected piglets is yet unknown. Further studies are needed to elucidate the epidemiology, the pathogenesis of the disease, the host-virus interaction and the exact routes of transmission are necessary. Future studies should also focus on virus monitoring especially in gilt acclimation protocols in order to help prevent the infection of the naïve animals during their gestation period, preventing the clinical signs in their offspring.

Abstract

9.0 English Abstract

Pestiviruses are highly variable RNA viruses within the *Flaviviridae* family causing economically relevant diseases of different kinds of livestock. A newly identified Pestivirus, the atypical porcine pestivirus (APPV), was identified in the USA in 2015. Since then, different APPV outbreaks were detected in several European countries, in America, and in Asia as well. Studies have shown that pregnant sows inoculated with APPV positive blood- or tissue-suspension are farrowing piglets affected by A-II type congenital tremor (CT). CT is characterized by a typical shaking of the head and the limbs of newborn piglets and is often complicated with splayed legs. Whereas the shaking itself is not the direct cause of death, it can prevent the piglets from proper nutrition, resulting in a decrease of growth rate and even death by starvation.

Recently, numerous pigs of different Hungarian herds were showing signs of CT and therefore they have been investigated for the presence of APPV by our research group. The aim of my research was to detect the virus in Hungarian swine herds, to characterize the strains and compare them to each other and to previously published sequences that can help in cognition of their origin, spread, and genome evolution. The second objective was to localize and visualize the virus in various tissue samples.

To do so, various tissue samples were obtained from four affected piglets, that were necropsied in 2017. One part of the samples were kept in –80 ºC for PCR-based virus detection, the other part was fixed in formalin and embedded in paraffin (FFPE). We isolated RNA from the frozen samples and detected the virus by conventional RT-PCR. We performed a novel RNA *in situ* hybridization method (RNAscope) on slides prepared from the FFPE samples to show the tissue localization of the virus.

Based on recent scientific studies and the results of our research group, APPV is mostly localized in the inner granular layer of the cerebellum, which is reported to be responsible for the clinical signs, and also in the ependymal and subependymal cells of the cerebrum.

10.0 Hungarian Abstract

A pestivírusok a *Flaviviridae* családba tartozó, igen változékony RNS-vírusok, amelyek világszerte jelentős gazdasági károkat okoznak különböző haszonállat-állományokban. 2015-ben az USA-ban azonosították a *Pestivirus* nemzetség egy új tagját, az atipikus sertés-pestivírust (APPV), amit azóta számos európai országban, Amerikában és Ázsiában is kimutattak. Fertőzéses állatkísérletekben megfigyelték, hogy APPV-tartalmú vérrel vagy szövet-szuszpenzióval megfertőzött vemhes kocák utódai az A-II típusú reszketőkór (congenital tremor, CT) tüneteit mutatták. A reszketőkórt az újszülött malacok fejének és végtagjainak remegése jellemezi, amely gyakran szétcsúszó lábakkal társul. Bár a remegés önmagában nem okoz közvetlenül elhullást, súlyos esetben megakadályozhatja a malacok megfelelő táplálkozását, amely a növekedési ütem csökkenéséhez és akár eléhezéshez is vezethet. A közelmúltban kutatócsoportunk több magyar sertésállomány esetében azonosította az APPV-t reszketőkórban szenvedő malacok szövet- és vérmintáiból. Kutatásom célja egy magyar állomány reszketőkórban szenvedő malacaiból származó szövetmintákból azonosítani a vírust, részleges szekvenciameghatározás után filogenetikai elemzés útján összehasonlítani a magyar törzseket az adatbázisban található más APPV-törzsekkel, amely segíthet eredetük, terjedésük és genom-evolúciójuk megismerésében. Ehhez 2017-ben az érintett állományból négy reszketőkór tüneteit mutató malacot boncoltunk fel, melyekből 9–9 szövetmintát vettünk. Egy részükből RT-PCR-módszerrel azonosítottuk a vírust, a másik részüket formalinban rögzítettük és paraffinba ágyaztuk, metszeteket készítettünk, majd egy RNS-alapú *in situ* hibridizációs módszerrel, RNAscope-pal vizsgáltuk a vírus jelenlétét a szövetekben. A legfrissebb tudományos vizsgálatok és kutatócsoportunk eredményei alapján az APPV elsősorban a kisagyvelő granuláris rétegének belső részének idegsejtjeiben található meg, amely feltételezhetően a klinikai tünetekért felelős. Ezen túlmenően számos egyéb szervben azonosítottuk a vírus genetikai állományát mindkét említett mórszerrel.

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