Project closing report

Most of the results will be published in the coming months, so the volume of the final report is longer, describing in detail the unpublished data.

Detection and analysis of emerging porcine parvovirus infections

New, previously unknown viruses belonging to the family Parvoviridae are often recognized in recent years in various animal species and humans alike. This is due to inter alia, the rapid development of molecular biological methods, because the sequence independent polymerase chain reaction methods allowed the detection of viruses, which identification was not possible with conventional diagnostic methods.

Several members of the Parvovirinae subfamily cause infections manifested by clinical symptoms, such as the porcine parvovirus type 1 (PPV1) related reproductive disorders (stillbirth, mummification, embryonic death and infertility, SMEDI), the Canine parvovirus type 2 related enteritis and lymphopenia, or the Feline parvovirus caused panleucopenia, to mention only the best known syndromes. However, most of the parvoviruses can be detected as subclinical or asymptomatic infection.

The virions of parvoviruses are non-enveloped and contain single-stranded, linear DNA genomes of approximately 5-6 kilobases (kb). Based on the current taxonomic classification (International Commitee on Taxonomy of Viruses, ICTV, http://ictvonline.org/virusTaxonomy.asp) the parvoviruses can be divided into two subfamilies within the family Parvoviridae: members of the Densovirinae subfamily infect various arthropods, while viruses belonging to Parvovirinae subfamily infect vertebrate animals. Based on the recent classification, Parvoviridae subfamily includes eight genuses, such as *Amdoparvovirus, Aveparvovirus, Bocaparvovirus, Copiparvovirus, Dependoparvovirus, Erythroparvovirus, Protoparvovirus* and *Tetraparvovirus*.

The PPV1 (*Protoparvovirus genus, Ungulate protoparvovirus 1)* has long been known which infect pigs and they have the ability to cause and occurs worldwide. Over the last years several new porcine parvoviruses were detected. First, the porcine parvovirus 2 (PPV2, *Tetraparvovirus* genus, proposed name Ungulate tetraparvovirus 3) has been described, by accident during a survey for hepatitis E virus (HEV) in samples originated from Myanmar. Another newly discovered member of porcine parvoviruses is porcine hokovirus, identified in

Hong Kong in 2008. This virus was nominated by the authors in several names (the most common is the PPV3), the current proposed classification is *Tetraparvovirus* genus Ungulate tetraparvovirus 2. Recently, further new porcine parvovirus, the PPV4 was described in North-Carolina (USA, *Copiparvovirus* genus, proposed name Ungulate copiparvovirus 2) from porcine circovirus type 2 (PCV2) disease affected pigs samples collected during 2005. The exact geographic distribution of these newly discovered porcine parvoviruses is not known yet, but they were also detected in Asia, in Europe, and in United States in domestic pigs and in wild boars, suggesting a perhaps worldwide presence. The Transylvanian and Chinese results suggest the spreading PPV3.

The first members of the *Bocavirus* genus were detected in samples from calves with diarrhoea in 1961 (Bovine parvovirus, BPV) and from the faecal sample of a dog in 1970 (minute virus of canine, MVC). An acute respiratory tract infection was described in children in 2005 and in the background of this a new parvovirus was detected, which was similar to BPV and MVC. The virus was named human bocavirus (HBoV, from **bo**vine and **ca**nine). Recently, other three HBoVs (HBoV2, HBoV3 and HBoV4) had been discovered in respiratory and enteric infections in humans. A porcine boca-like virus (PBo-likeV) was described in Sweden, using random multiple displacement amplification (MDA) and large-scale sequencing and it was reported recently in wild boars in Romania. Beyond Europe PBo-likeV was also identified in China.

Also in China, faecal samples from healthy piglets (<15 days of age) were examined with a sequence independent single primer amplification method suitable for the detection of unknown sequences. With this method, four new porcine parvoviruses were identified. According to the genome organization and sequence analysis, the sequences belonged into the *Bocavirus* genus. Among these porcine bocaviruses (PBoV), two nearly full length genomes were determined (PBoV1 and PBoV2). From the other two PBoVs (labelled 6V and 7V), only 2407 and 2434 nucleotides were obtained. These two sequences did not group with PBoV1 and PBoV2; they shared less than 56% similarity with them and had less than 50% similarity with the PBo-likeV. Based on phylogenetic analysis PBoV-likeV, PBoV1, 2, 6V and 7V formed three different clusters within the *Bocavirus* genus.

Recently, two other porcine bocaviruses (PBoV3 and PBoV4) were described in Northern Ireland, from porcine circovirus type 2 (PCV2) disease affected pigs.

The aim of this OTKA project was to:

1. Studies of the detection, prevalence, incidence and evolution of porcine parvoviruses will be completed by the phylogenetic analysis of parvovirus genomes collected in Central Europe.

2. Development of antibody detection systems (ELISA) and quantitative real-time PCR systems, specific for different, newly described porcine parvoviruses.

3. Identification of potential cell and organ targets of the newly described porcine parvoviruses.

4. Finding relationship among the detected viruses, observed symptoms and pathological changes.

First of all, the Hungarian epidemiological status was examined by PCR method. A variety of samples (215 faecal samples, 111 blood serum samples, 38 lungs, 22 tissues of different organs like liver, kidney, spleen, lymph nodes, 4 aborted foetuses and 2 sperm samples), altogether 392 from 57 swine herds of different regions of Hungary were tested. The samples arrived for different routine diagnostic examinations during 2006-2011. All of the examined parvoviruses were present in Hungary. Thanks to the widespread vaccinations against classical porcine parvovirus, PPV1 was detected only in one Hungarian swine herd. PPV2 was present in 15, PPV3 in 19, PPV4 in 13, PBo-likeV in 6, PBoV1 and PBoV2 type viruses in 8 and the 6V and 7V type parvoviruses were detected in 6 of the 57 examined herds. Based on sequence analysis (shown later) the PBoV1 and PBoV2 specific primers and the 6V and 7V specific primers amplified highly variable genomic areas of these viruses indicating that the simple distinction of PBoV1 and PBoV2 or 6V and 7V was not accurate enough for these sequences, therefore we will refer to them from this point as PBoV and 6V7V. Two herds were co-infected with PPV2 and PPV4, one with PPV4 and PBo-likeV, and one with PPV2 and PBoV. One swine herd was infected simultaneously with PPV2, PPV3 and PPV4, another one with four different porcine parvoviruses (PPV2, PPV4, PBoV and 6V7V). Two herds were infected with five different parvoviruses, one of these with PPV2, PPV4, PBo-likeV, PBoV and 6V7V, and one swine herd with PPV3, PPV4, PBo-likeV, PBoV and 6V7V. Among the 392 samples, two samples were positive for PPV1, 25 for PPV2, 38 for PPV3, 25 for PPV4, 6 for PBo-likeV, 19 for PBoV, 7 for 6V7V. The positivity rate was similar for each year for all of the novel porcine

parvoviruses except for PPV3, where the number of positive samples increased from 6% to 15% during the period examined. Several samples were found to contain two of these viruses, indicating a double infection.

Up to our PPV2 was not detected outside Asia, but we proved that it was also prevalent in Hungary, with 93-100% genetic identity among the viruses, based on a short, conserved region of known PPV2 sequences. PPV2 was detected not only in blood serum, but in faecal samples and also in different organ samples, mostly in lungs and liver. PPV3, similarly to PPV2 could be detected in blood serum, in faecal samples and also in lungs, lymph nodes and liver. PPV4 was detected in 13 of the 57 swine herds, so it is considerably wide-spread in Hungary. The virus was present mostly in faecal samples, but also in blood samples, boar semen and in three out of four aborted foetuses. There were no other known pathogens (Porcine circovirus 2 PCV2, PPV1, porcine reproductive and respiratory syndrome virus PRRSV, pseudorabies virus) in these PPV4 positive samples. Our findings confirmed previous results showed that PPV4 may play some role in reproductive disorders, but to prove these further experiments will be needed.

In Hungary only 6 samples from 6 different swine herds were positive for PBo-likeV. In five faecal samples and only in one lung tissue sample was PBo-likeV detected. Two PBo-likeV positive faecal samples were also positive for PCV2 and the single positive lung sample was negative for PCV2. These data suggest the PBo-likeV is not such an important risk factor at least in Hungarian swine herds. PBoV type viruses were detected more frequently in the samples of this study (4.8%) than the 6V7V type viruses (1.8%), but with lover frequency than in China. Similarly to Chinese examinations the PBoV and the 6V7V types were present mostly in stool samples, but not only in young pigs, guilts and fattening pigs were also infected with these viruses. PPV4 and PBo-likeV sequences showed genetically uniform pictures suggesting that the adaptation of these viruses to swine were in advanced state or already reached a comfortable level. For PPV3 and most of the PPV2, PBoV and 6V7V sequences higher genetic variations were shown. The higher divergence and the deletions or insertions of nucleotide triplets in PBoV may indicate that these newly described parvoviruses were in the process of adaptation. Recombination of the genome of parvoviruses can also play an important role in adaptation and in virus evolution, our results shows the possible sites of recombination in PBoV sequences that were identified.

After these results, we examined the evolution of novel porcine parvoviruses. To this purpose, in addition to Hungarian samples, Central and Eastern European domestic pig (Romania, Poland, Serbia and Croatia) and free-living wild boar (Transylvania) samples were analysed.

Our study was the first that explored the molecular characteristics of porcine parvovirus strains circulating in wild boar populations and because of the limited number of PPV1 sequences (all of them of domestic pig origin) available worldwide, provides important new findings about the phylogeny, molecular epidemiology and evolutionary dynamics of parvovirus in both domestic and wild hosts. Bayesian inference of phylogeny showed that the domestic and wild boar PPV1 strains could be grouped in six groups/clusters (A–F) based on the complete VP1/VP2 genes, and in eight groups (A–H) based on the extended partial VP1/VP2 sequences. The clusters A–E within trees of both datasets characterized by a high posterior probability ($\geqslant$0.90) indicate that the members of each cluster had descended from a well-defined common ancestor. Interestingly, the wild boar strain WB-716 diverged from other members of cluster A and showed a somewhat different evolution pattern which may suggest that this strain could be a wild boar specific porcine parvovirus. To support this hypothesis, further retrospective evolutionary studies are required both in domestic pigs and wild boars. Considering that all of the wild boar PPV originated from a relatively small, well-defined area (the entire region of Transylvania; Romania), phylogenetic data indicate that PPV1 in wild boars is more diverse compared to domestic pig PPVs from the same region and the entire world. This fact could be explained by the presence of several factors such as the dissemination of the same virus strain in domestic swine herds by mixing animals of different sources and, most importantly, extensive vaccinations that may contribute to the selection pressure on PPV1 resulting in a homogenous viral population. In case of wild boars the absence of these factors and the limited contact with domestic sources may contribute to the higher diversity of PPV1 in the wild habitat. Also, phylogeny analysis showed that the circulating PPVs in wild boars are partially different and independent in comparison to the domestic pig viruses. It is interesting to note that in the area corresponding to the wild boar sampling region we found the exclusive presence of a 27a-like strain in the domestic pig populations (strain 6R) that may lead to different assumptions. Vaccination may provide protection against strains circulating in domestic pigs, except 27a-like strains. This fact is supported by previous cross-neutralization studies performed against vaccine strains (NADL-2 and IDT) that showed low neutralization activity against the PPV-27a strain, indicating incomplete. The extensive use of several-decade-old vaccine strains might be the reason for the emergence of 27a type viruses. Studies have reported

that phenotypic differences, tissue tropisms and other viral properties are determined by few residue changes in the VP1/VP2 proteins. These amino acids differing in the VP1/VP2 protein when comparing the NADL-2 with the Kresse strain (Thr195 → Ser, Ile365 → Thr, Asp528 → Gly, His533 → Gln, Ser586 → Pro, and Arg715 → Lys) were also detected in wild boar PPVs. Notably, we found the existence of some specific residues that are able to differentiate the aforementioned six clusters. Mutations involving residue Thr102 → Ser (VP1) have not previously been reported for PPV1 in the literature, but are specific for cluster A. We also described two additional clusters (C and D), one of them (cluster D) having a previously not described substitution Ser436 → Ala at one critical mutation point involved in tissues tropism and differentiation of clusters. The majority of the new residue substitutions located mainly in the protein loops described very recently were found here to be involved in the differentiation of clusters A and B. These regions play a role in antigenicity and/or contain phenotypically important residues. The biological consequences of these mutations are not clear yet, although it has been observed that the new variants (strain 27a) have acquired definite advantages in comparison with older PPV1 strains due to lower affinity to neutralizing antibodies. The molecular clock analysis combined with the phylogenetic trees of the VP1/VP2 datasets presented here suggest that the main divergence times of the clusters occurred in the last 20–60 years. In addition, the divergence of cluster A that occurred approximately 120 years ago, represent the most distant and different cluster including the possible wild boar specific PPVs.

The prevalence of novel, newly discovered PPVs in Transylvanian domestic pigs and wild boars were detected by PCR. Tissue samples of a total of 120 domestic pigs and 842 wild boars from the entire region of Transylvania were analysed for the presence of PPV2, PPV3, and PPV4 by PCR. It was observed that 25% (30/120) of all examined domestic pig samples and 10.3% (87/842) of wild boars were positive for PPV2. In a previous study, we showed that the overall prevalence of PPV3 by PCR in Transylvanian free-living wild boars was 35% (295/842) while in a later study the prevalence of PPV3 in domestic pigs was lower, 17.5% (21/120). The presence of PPV4 remained at a very low level in wild boars 0.95% (8/842), whereas in domestic pigs it was 10% (12/120) in accordance with previous. The majority of PPV positive domestic pigs (62%, 39/63) were infected with two or three viruses tested. Co-infection of PPV2 and PPV3 was the most prevalent (79%, 31/39) in domestic pigs, while single infection was the most prevalent type in wild boars accounting for 54.1% (213/390) of infections. Co-infection of PPV2 and PPV3 in wild boars was observed as the second most prevalent type of

infection (95%, 169/177), while single infections with PPV4 were not observed in either group of animals.

The sequence and phylogenetic analysis of PPVs cap genes resulted five PPV2, 10 PPV3 and four PPV4 DNA fragments from wild boars and 5, 8 and 4 of those fragments from domestic pig samples representing the complete cap gene were amplified and sequenced to be compared with the published sequences deposited in the GenBank. Levels of nucleotide and amino acid similarity of PPVs between wild boar and domestic pig samples included in our study, and in comparison with the ones from the GenBank database were 93.7–98.4%, 93.9–96.8% (PPV2), 97.8–100%, 98.1–99.5% (PPV3), 98.4–99.5%, 98.6–99.8% (PPV4) at nucleotide level, and 94.1–98.5%, 93.7–99.1% (PPV2), 98–100%, 98.1–100% (PPV3), 98.2–99.8%, 97.9–100% at amino acid level (aa), respectively.

Based on complete cap gene, the PPV2 clade is grouped into two strongly supported clusters. One of them (A) is split into two distinct subsets, one formed exclusively by Romanian domestic pig sequences, while the other one by sequences of mixed origin. The members of this cluster are characterized by amino acid replacements Ile784Met, Asn788Ser and Glu796Gln. Cluster B consists only of wild boar PPV2 and the first PPV2 detected in Myanmar. The PPV3 clade can be grouped into four highly supported clusters (A–D): cluster A contains 2 sequences detected in UK domestic pigs in 1999 and one Romanian PPV3 of wild boar origin from 2006; cluster B is divided into two subsets, one of which consists only of recently detected Romanian domestic pig PPV3, while the other grouped German and Romanian wild boar PPV3 and UK domestic pig origin sequences; cluster C also shows subset patterns with two distinct UK domestic pig origin PPV3 subsets and a divergent Romanian wild boar PPV3, while cluster D contains two highly divergent Romanian wild boar PPV3 and 3 distinct subsets. The members of each cluster mentioned above exhibit some specific amino acid replacements Ala321Val, Leu332Arg (A), Val740Gly (B), Ile383Val (C), Leu105Ile (D). The clade of PPV4 formed a well-supported cluster where domestic and wild boar sequences from our study are grouped mainly together, while the USA and Chinese PPV4 were grouped separately.

Our study was the first report describing the presence of porcine parvovirus 2 and 4 within wild boar populations. Furthermore, our findings suggest that these newly discovered parvoviruses co-circulate both in domestic pig and wild boar populations and showed that they may harbour multiple novel parvoviruses. High prevalence of novel PPVs, except PPV4, in both hosts indicates endemic circulation of these viruses. Comparative phylogeny of PPVs have placed

PPV2 phylogenetically closer to PPV1, while PPV3 and PPV4 are located more distantly. Our PPV2 phylogenetic analysis divided this virus into two highly supported clusters. Although frequent recombinations observed in PPV2 can potentially impact phylogenetic relationships and almost each strain studied here exhibited recombination history, the characteristic amino acid replacements that define these clusters were located in the non-recombinant region of the gene. It is worth noting that cluster A (PPV2) consisted only of wild boar strains and the first detected PPV2 in domestic pig. Given that PPV2 strains of cluster A circulating in wild boars originated from years 2006 to 2011, it may suggest that these strains could be wild boar specific PPV2, especially WB-763S that diverged from other ancestors of cluster A and showed a somewhat different evolution pattern. Phylogenetic data indicate that wild boar PPV2 originating from a relatively well-defined area is more diverse compared to domestic pig PPV2 from the same region. Similar phenomenon was observed for PPV3 where higher clustering patterns evolved. The high rate of PPV2 and PPV3 variability is shown by the existence of a number of viral variants simultaneously present within a single host, and of different viral strains identified at intra-host level. There are no significant clustering patterns in the phylogeny of PPV4, probably due to the fact that the cap gene of this virus is highly conserved and shows high homology among domestic and wild hosts. Our phylogenetic analysis did not reveal evidence of relationship between the phylogenetic clustering of the strains and the geographic origin of the viruses. However, considering that all of the novel wild boar PPVs (except BW2117 and Sa15 PPV3) originated from a relatively well-defined area (entire region of Transylvania, Romania), phylogenetic analysis indicates that PPV in wild boars is more diverse compared to domestic pig PPVs from the same region as it is in case of PPV1. The results of our analyses indicated that novel PPVs originated within approximately the last 70 years. The TMRCA for PPV2 (37 years before present, ybp) was closer to that of PPV4 (25 ybp) and more recent than that for PPV3 (71 ybp). A relatively high evolutionary rate similar to those commonly found in RNA viruses was observed for PPV2 capsid gene ($\sim 8.23 \times 10^{-4}$ subs site^{-1} year^{-1}) and also for PPV3 ($\sim 3.86 \times 10^{-4}$ subs site^{-1} year^{-1}) and PPV4 ($\sim 4.53 \times 10^{-4}$ subs site^{-1} year^{-1}). Our results therefore suggest that the high evolutionary rate of PPV2 cap gene is probably influenced by frequent recombination.

Analysis of selective pressures acting on the capsid gene suggests that they are mostly subject to purifying selection with only two codons in PPV2 and PPV3, and one in PPV4 being positively selected under all five implemented methods. The newly designed likelihood method FUBAR showed that the number of positively detected sites under REL analysis could be

overestimated. Using another very recently designed method, MEME, we found several episodic and pervasive positive selection sites among all regions of capsid genes of these novel PPVs. Using branch-site specific analysis we observed that the diversification pressure was strong in some branches of each studied novel PPVs. Unfortunately, there are no data available on the functional domains of the cap gene of the newly discovered PPVs. The positively selected sites of PPV2 observed only in domestic host showing alternative replacements of amino acid 269 and 1010, suggest a role of these residues in the adaptation of PPV2. It is interesting to note that the positively selected Pro233Ser mutation detected only in wild boar origin PPV3 capsid protein sequences that may provide some adaptive advantage to the new host is located near to the conserved Ca^{2+} binding loop of secreted phospholypase A_2 (PLA_2) domain. This domain is known to play a major role in the infectivity of PPV1. The conserved motifs YXGXG and HDXXY corresponding to the consensus sequences of the conserved Ca^{2+} binding loop and catalytic site of secreted PLA_2 domain were also found in the capsid protein sequence alignments of the parvoviruses in our examinations. This suggests that the capsid protein of these newly discovered porcine parvoviruses carry a PLA_2 domain and may play a similar function in the infectivity as in case of other parvoviruses.

Thanks to international collaboration, we could examine and analysed faecal specimens from Africa. Our study was the first to report evidence of the circulation of bocaviruses in Africa and contributes to our understanding of the impact of globalization on the dispersal of new and emerging viruses. In Cameroonian samples, high prevalence and remarkable genetic diversity within the identified parvoviruses and, particularly, within bocaviruses was observed. PPV4 was the most prevalent virus (20%), followed by PBoV3 (18%), PBoV4 (18%), PBoV5 plus 6V/7V (16%), and PBoV1 plus PBoV2 (6%). The frequency of mixed infections with various combinations of these virus species reached 20%. Genetic analysis of the identified viruses showed that the capsid gene of PBoV1 and PBoV2 strains shared up to 91% and 94% nt sequence similarities to reference PBoV1 and PBoV2 strains, respectively. The identified PBoV3 and PBoV4 strains shared $\leqslant$95% and $\leqslant$98% nt identities with reference PBoV3 and PBoV4 strains, respectively, along the NS gene, whereas the PBoV5 strains shared 86% nt identities with Hungarian and 87% nt identities with Chinese PBoV5 strains along the capsid gene. In addition, a single PBoV5-like strain shared $\leqslant$71% nt sequence identity with other PBoV5 strains.

In conclusion, our studies provides important new results about the phylogeny and evolutionary dynamics of porcine parvoviruses in their evolution in domestic and wild hosts. The detection of high rates of evolution and genetic diversity of PPVs in wild boar populations emphasize the need to perform additional investigations in order to clarify the epidemiological role of wild boars in PPVs infection and their possible role as sources of new variants of PPVs.

The newly described emerging PPVs are frequently detected in different clinical signs affected pigs suggesting a pathological role of the virus at least as co-factor (mostly in porcine respiratory disease complex). In order to confirm the possible pathogenic role of PPV2 it is necessary to develop diagnostic methods for the *in situ* detection of the virus in tissue environment and also to detect and monitor the antibody response to PPV2 in context with the appearance of clinical signs.

One of the aim of our work was to develop ELISA methods suitable for the detection of new, emerging PPV specific antibodies. Using this method we measured the specific humoral immune-response against PPVs in infected swine herds and compared the antibody levels with the presence of the virus in tissue and serum samples. We also developed in situ detection methods, suitable to detect these PPVs in tissue environments. These works will be publish in next months, the manuscripts are under preparation, for this, these examinations are written in more details:

Examination of serological profile:

The examinations carried out in PPV2, PPV3 and PPV4, but to date, the data are analysed only with PPV2.

Materials and methods

Sample collection

Lung samples were collected from four different Hungarian swine herds to determine the presence of PPV2. Based on the results, one PPV2 positive swine herd was selected and examined further. Altogether 185 serum samples were collected from a PPV2 positive swine herd, from different age groups. Fifty-five samples were collected (5x3 samples/litter) from 2, 7, 14, 21, days old piglets and their sows, and 28, 36, 43, 57, 90, 120 and 150 days old growing and finishing pigs. The serum samples were stored at -20 ^{o}C until further examinations.

Quantitative PCR

For PPV2 copy number quantification the capsid protein coding ORF2 was amplified with PPV2PacI (CGGTTAATTAATTATACACGATGAGCGCGT) and PPV2NotI (GATTAGCGGCCGCCATGAGCGCTGCCGA) primers and cloned into the pBacPAC9 (Clontec) plasmid digested by *Pac*I and *Not*I (Thermo Scientific). The insert bearing plasmid was propagated in TOP10 cells (Invitrogen), and purified by EZ-10 Spin Column Plasmid DNA Minipreps Kit (Bio Basic), based on the user manual. The presence and accuracy of the PPV2 fragment in the plasmid was confirmed by sequencing. The amount of purified DNA was determined by NAS-99 NuDrop Micro-volume Nucleic Acid spectrophotometer at 260 nm, and the copy number was calculated (URI Genomics & Sequencing Center, http://cels.uri.edu/gsc/cndna.html). To determine the copy number of PPV2 nucleic acid in serum samples, ten-fold serial dilutions were prepared from the plasmid and amplified as a control by qPCR parallel with the samples.

Total nucleic acid was extracted from all of the 185 serum samples with GeneJET Viral DNA and RNA Purification Kit (Thermo Scientific), according to the given protocol. PPV2 DNA was amplified with PPV2AF and PPV2AR primers suitable for the detection of both the Myanmar-type and the Cnvirus-type sequences, in an Eppendorf realplex2 Mastercycler ep gradient S thermal cycler instrument. The reaction mixture contained 1µl of each primers (25pM), 12.5µl of Maxima SYBR Green qPCR Master Mix (Thermo Scientific), 2µl sample and distilled water to a final volume of 25µl. The nucleic acid amplifications were as follows: UDG pre-treatment for 2 min at 50ºC, preheating for 10 min at 95ºC followed by 40 cycles at 95ºC for 15 s, 60ºC for 30 s, 72ºC 30 s. The amplifications were completed with melting curve analysis.

PPV2 antigen preparation

Partial, 792 nucleotide long capsid protein (264 amino acids) coding sequences were amplified with the PPV2-EcoRI-2035F (GGAATTCTTTCCCAGTCTCGAACC) and PPV2-XhoI-2826R (GGCTCGAGCACCTGCGGCTGCAT) primers specific for both the Myanmar-type and Cnvirus-type viruses. The PCR products were cloned using *Eco*RI and *Xho*I endonucleases (Thermo Scientific) into the modified pBAD/Thio-TOPO vector (Invitrogen). The insert containing plasmids were propagated in TOP10 cells (Invitrogen), purified by EZ-10 Spin Column Plasmid DNA Minipreps Kit (Bio Basic) and confirmed by sequencing. The partial

PPV2 capsid proteins were produced in BL cells. Briefly, one colony of plasmid-containing cells was cultured overnight (37ºC) in 5ml LB Medium (containing 100µg/ml ampicillin, Fluka). The overnight cultured cells were grown for one hour in 100 ml fresh, pre-warmed (37ºC) LB Medium (containing 100µg/ml ampicillin) after which one ml of 20% L-Arabinose (Sigma-Aldrich) was added to induce the gene expression. The induced cells were incubated for 4 hours, harvested by centrifugation and stored at -20 ºC until purification. Before and after the induction, 1 ml of each culture was taken and the protein production was monitored by SDS-polyacrylamid gel electrophoresis (SDS-PAGE).

The produced partial PPV2 capsid proteins were purified by Ni-NTA Agarose (Qiagene) under denaturing conditions (using guanidine lysis buffer), based on the protocol in "The QIAexpressionist A handbook for high-level expression and purification of 6xHis-tagged proteins" (http://kirschner.med.harvard.edu/files/protocols/QIAGEN_QIAexpressionist_EN.pdf). The purified proteins were analysed by SDS-PAGE. The amounts of proteins were determined using the NAS-99 NuDrop Micro-volume Nucleic Acid spectrophotometer at 280 nm.

ELISA

The purified antigens were diluted to 5µg/ml in phosphate buffered saline (PBS) and ELISA plates (Nunc, Immuno Medisorp FH) were coated with 100µl/well of the diluted antigen for one hour at 37ºC. After the incubation, plates were washed three times with PBS containing 0.05% Tween 20 (PBST) and blocked by 200µl of 1% bovine serum albumin and 0.05% Tween 20 diluted in PBS (BSAT) for two hours at 37ºC. After washing three times, sera (1µl diluted in 100µl BSAT) were added and incubated for one hour at room temperature. After subsequent wash with PBST, the plates were incubated with peroxidase-conjugated anti-swine IgG (whole molecule, Sigma-Aldrich, diluted 1:30.000 in BSAT) for one hour at room temperature. Following the three washing steps, chromogenic substrate (3,3′,5,5′-Tetramethylbenzidine, Sigma-Aldrich) was added and incubated in dark for 20 min. The enzymatic reaction was stopped by 50µl of 2N H_2SO_4 and the colour reaction was detected in an ELISA reader at 450nm.

Western-blot analysis

Non-induced and induced, empty and partial PPV2 capsid gene bearing pBAD/Thio-TOPO vector containing cell lysates (10µl) and purified partial PPV2 capsid proteins (1µg) were separated by SDS-PAGE, and the separated proteins were transferred to Nitrocellulose membrane (Kisker). The membranes were blocked with BSAT for one hour at 37ºC and washed with PBST, then incubated with 10µl porcine serum samples -diluted to 10 ml in BSAT- for one hour at 37ºC. The unbound antibodies were removed by washing with PBST and the membranes were incubated for one hour at 37ºC with 10ml peroxidase-conjugated anti-swine IgG (whole molecule, Sigma-Aldrich), diluted to 1:5.000 with BSAT. After being washed with PBST, the membranes were incubated with the chromogenic substrate (3,3′-Dimethylbenzidine, Sigma-Aldrich). After 15 minutes, the enzymatic reaction was stopped, removing the substrate by washing the membranes under tap water.

Results

Detection of viraemia

The PPV2 detected in the examined swine heard was of the Myanmar-type. According to qPCR results viraemia was detected in every age group mostly below $6.25x10^3$ copies/ml. Higher virus loads were measured in 57 days old pigs where 6 of the 15 serum samples were highly PCR positive, containing PPV2 genomic copies of $4.20x10^4$/ml, $5.45x10^4$/ml, $9.48x10^4$/ml, $4.48x10^5$/ml, $5.06x10^5$/ml and $7.14x10^5$/ml. In the 90 days old age group 3 samples out of 15 were highly PCR positive, containing $2.19x10^4$/ml, $3.76x10^4$/ml and $1.16x10^5$/ml copies and among the 120 days old pigs only one sample was highly PCR positive: $1.53x10^4$/ml PPV2. The $5.45x10^4$/ml, $9.48x10^4$/ml and $7.14x10^5$/ml copy number containing samples were negative in ELISA, the others carried PPV2 specific antibodies.

Examination of PPV2 protein produced in bacteria

264 amino acid partial PPV2 capsid protein coding 792 nucleotide long sequences from the Myanmar-type and Cnvirus-type viruses were cloned into a modified pBAD/Thio-TOPO vector. These viruses differ from each other in 14 amino acid residues within the expressed region. The protein production of induced and non-induced bacterial cells was examined by SDS-PAGE. Both types of PPV2 partial capsid proteins were produced in sufficiently high amount to allow purification. The purified proteins were also examined by SDS-PAGE. The reactivity and purity of produced proteins were analysed through Western-blotting. The SDS-

PAGE separated cell lysates of induced and non-induced, empty plasmids and PPV2 genomic insert carrying plasmids containing bacteria, as well as purified proteins were transferred to Nitrocellulose membranes. Based on the results of ELISA examinations, Western-blot analysis was performed with PPV2 positive and negative porcine serum samples. None of the empty plasmid and non-induced plasmid carrying cells reacted with these sera, only the induced PPV2 partial capsid protein containing cell lysates and purified partial PPV2 proteins reacted with pig sera that were positive in ELISA, and the negative serum did not show any reaction with these protein samples.

PPV2 specific antibody profile with Myanmar-type and Cnvirus-type antigens

To determine the PPV2 specific antibody profile of an infected swine heard, serum samples were collected from different age groups (sows, 2 days old to 150 days old pigs) and examined with the ELISA developed here. The serum samples of two days old piglets had higher antibody titres than their sow. The maternal antibody titre decreased until 14 days of age, PPV2 specific antibody levels started to rise between 36 to 43 days of age, and the peak was measured between 57 and 90 days of age. In order to find out the difference between the partial Myanmar-type and Cnvirus-type antigen, serum samples of the Myanmar-type PPV2 infected swine herd were examined with both types of the antigen. The antibody levels measured on the two antigens were very similar, but mildly stronger reaction was detected with the homologous, Myanmar-type antigen at 36 and 43 days of age, and clearly stronger reaction was measured at 90 days and over.

Discussion

Several new, emerging parvoviruses were discovered in pigs during the recent years and according to the so far limited number of the reports these viruses are widespread in pig populations around the world. Clarification of the pathogenic role of the new parvoviruses including PPV2 is still pending mainly due to the lack of reliable *in vitro* virus propagation techniques for experimental infections. Swine herds are frequently infected not only with PPV2, but simultaneously also with one or more other pathogens, like PCV2, PRRSV, Swine influenza virus (SIV), *Mycoplasma hyopneumoniae, Actinobacilus pleuropneumoniae, Pasteurella multocida* and other microorganisms that prevent or make very difficult to clarify the pathogenicity of PPV2. Fortunately *in situ* detection methods and serological techniques at least in part may be of help to determine the importance of a PPV2 infection. In this study, we

developed a serological method to examine the specific humoral immune response against PPV2, and determined the PPV2 specific antibody profile of on affected swine herd. According to our best knowledge, this is the first description of serological examination of this virus infection. Based on our results, the piglets suckling colostrum from their PPV2 positive sows and adsorbing high amounts of specific antibodies have very good passive, maternal protection. On the second day of life PPV2 specific maternal antibody level is higher in piglets then in their corresponding mothers, but this protection decreases rapidly. The increase of PPV2 specific antibody level starts after 36 days of age and calculating with the usual 7 to 10 days required for the primary immune response of B lymphocytes the estimated time of infection is suggested to be earlier than or between 28-36 days of age. In the examined swine heard, respiratory signs were observed around 36-43 days of age and by 57 days of age these clinical signs disappeared. Along with this, after 36-43 days of age a rapid rise of PPV2 specific antibody levels could be observed, resulting high amounts of antibodies in the 57 days old group. The serum samples were also tested for PCV2 and SIV specific antibodies (data not shown), but the rise of PCV2 specific antibodies was observed only after 120 days of age and the increase of SIV specific antibodies was not detected in pigs over 57 days of age. The examined swine heard was free of PRRSV, and the bacteriological examinations for common respiratory pathogens were also negative. These data suggest that PPV2 may have played a role in the development of the respiratory signs around 40 days of age. This assumption is supported by the frequent detection of PPV2 in pigs exhibiting respiratory diseases. In our previous study in Hungary 10.5% of the examined lung samples were positive for PPV2. In a Japanese swine herd, all of the 69 examined tonsil samples, collected from unhealthy 8 to 900 days old pigs were PPV2 positive. In the USA the highest levels of PPV2 nucleic acid were present in lung samples originating from nursery and grow-finish pigs with a history of respiratory disease, covering the age of pigs, where the respiratory clinical signs and PPV2 specific antibody rising was detected in our study. Based on a retrospective study PPV2 was present with high prevalence in archived North American lung samples from 1998 not only in PCVAD affected (33.3-55.6%) but also in PCV2 negative pigs (20%). The prevalence rate of viraemia in samples collected between 2006 and 2013 in North America was 27.3-41.4% in PCV2 positive and 33.3% in PCV2 negative sera. Previously in China, PPV2 was identified in 9.66% of serum samples of PCV2 and PRRSV caused disease affected pigs, 3 weeks prior the disease onset. Similarly in the USA, the peak of PPV2 viraemia was detected at 15 weeks of age, 2 weeks before the development of PCVAD. In our study, low level of viraemia was detectable in every age group, but the peak was measured in 57 days old pigs, earlier than in previous studies. In this age group, 3 out of 6

highly PCR positive animals had no PPV2 specific antibodies. It is likely that the antibodies produced by this age in increasing quantity and quality started to eliminate PPV2 from the blood stream. After the initial IgM antibody production the immune response normally produces IgG antibodies of higher affinity and larger quantity, and gradually these that fit better the PPV2 antigens result in a more effective neutralization of the virus and activation of different antibody-mediated immune-elimination mechanisms. Affinity maturation is also reflected in the data of our ELISA tests, as the more specific antibodies produced in later phase of the immune response showed stronger reaction with the homologous Myanmar-type antigen then the heterologous Cnvirus-type antigen. This relatively small difference however did not influence the usability of these antigens in the serological diagnostic test.

Taking into account the few studies about PPV2 the results of this study suggest a pathological role of PPV2 in respiratory disease of pigs, but to clarify the importance of this virus further, well designed studies including experimental infections are needed.

Examination of affected cell types:

In order to understand the possible pathogenic role of these newly discovered porcine parvoviruses, essential to find out which are the target cells and organs of these viruses. The aim of following study was to develop *in situ* polymerase chain reactions (*in situ* PCR) methods to detection of different PPVs in tissue environments. In addition, we also applied immunohistochemistry to determine the characteristics of affected cells.

Materials and methods

The samples were collected from a swine heard and from a slaughterhouse. From swine heard, 13 dead pigs were examined; their ages were 5 to 18 weeks old. The 13 slaughterhouse-origin lung samples were 5 months old. After the pathologic examinations, bacteriological and molecular biological examinations carried out to detection of microbes. The porcine parvoviruses adenoviruses and circoviruses were tested by PCR methods. The procine reproductive and respiratory syndrome virus (PRRSV) and swine influenza virus (SIV) examinations were carried out by immunohistochemical methods.

Tissue preparation for in situ methods

After post-mortem examination, lymph nodes and lungs were fixed in 10% buffered formaldehyde for 24 hrs at room temperature, dehydrated through different grades of ethanol and in xylene, embedded in paraffin blocks and stored at -20°C. Tissue was routinely stained with standard technique of H&E, examined under light microscope while type and frequencies of microscopic lesions were determined.

In situ polymerase chain reaction

The different PPV positive tissue section was dewaxed in xylene and rehydrated through different grades of ethanol. Dewaxed sections on DNase/RNase-free electro charged slides were incubated with 40µl Proteinase K (Dako) in 2ml Tris–HCl buffered saline (TBS) for 3 min at room temperature. The different PPVs specific direct *in situ* PCR was performed using specific primers, HotStart Taq Plus DNA Polymerase (2.5 units/reactions, Qiagene), and PCR DIG Labeling Mix (x µl/reactions, Roche). A total of 10 µl of PCR solution was added to the slides and covered. The PCRs were carried out in xx instrument. The cycling conditions were 95ºC for 5 min, followed by 18 cycles of 95ºC for 45 s, 58ºC for 55 s, 72ºC for 60 s, and final elongation step at 72ºC for 5 min. After the amplification, the coverslips were removed and the slides were

Immunohistochemistry to detect cell markers

A total of 3 primary antibodies, anti-human CD3, anti-SLAIIDQ and anti-lysozyme were used to specify immune cell types infected by PPV2-4. Immunohistochemistry (IHC) was performed with standard HRP conjugated detection system (Envision, Dako). Tissue sections were dewaxed with xylene and rehydrated through graded alcohols. Endogenous peroxidase activity was blocked by incubation with hydrogen peroxide 3% in 0.1 M Tris-buffered saline (pH=7.6) (TBS) distilled water for 30 min. After Pronase or heating pre-treatment, depending on the antibody used (see Table 1.), tissue sections were rinsed in TBS and incubated with 20% normal goat serum solution in TBS for 1 h at room temperature. All antibodies were overnight incubated at 4°C. Anti-rabbit labelled polymer was used for detection of CD3, SLAIIDQ and lysozyme for 1 h at room temperature. Sections were finally incubated in diaminobenzidine (DAB)–hydrogen peroxide solution for 5 min (Dako, Denamark). Slides were shortly counterstained in Mayer's hemtoxilyn, dehydrated, covered with a coverslip and microscopically examined. As positive control human lymph node and swine lymph node sampled from slaughtered health pig were used. As negative controls, irrelevant primary

antibodies at the same dilution were used in substitution of the specific antibodies. Immune response was observed in lungs and lymph nodes was graded as no immune cells or “0”, weak infiltration or “1”, moderate infiltration or “2” and severe infiltration or “3”.

Double staining *in situ* PCR/IHC

Double staining to detect PPV2-4 and B and T lymphocytes and macrophages was performed using previously described protocol for *in situ* PCR do detect PPV2-3 and IHC to detect CD3, SLAIIDQ and lysozyme. Double staining is performed in a both direction the forward direction, the first to detect nucleic acid (PPV2-4) and then antigen (CD3, SLAIIDQ and lysozyme) and in reverse direction the first antigen (CD3, SLAIIDQ and lysozyme) and then to detect nucleic acid (PPV2-4).

IHC- in situ PCR

The slides were submitted to *in situ* PCR to detect virus. After incubation with NBT/BCIP solution, slides were put in TBS while appearance of blue chromogen was checked under light microscope. Endogenous peroxidase activity was blocked by incubation with hydrogen peroxide 3% in TBS for 30 min. Tissue sections were rinsed in TBS and incubated with 20% normal goat serum solution in TBS for 1 h at room temperature. Forward IHC protocol was performed as identical like previously described with two exceptions. *Pronase* incubation was not performed since tissue was already incubating in citrate buffer pH6 for 20 min at 96°C *Pronase* in *in situ* PCR protocol.

In situ PCR -*IHC*

The slides were submitted to IHC to detect CD3, SLAIIDQ and Lysozyme. After incubation with DAB solution, slides were put in 100% ethanol while appearance of brown chromogen was checked under light microscope. Slides were proceed to *in situ* PCR protocol, like previously described.

As a control in each case was performed IHC or in situ PCR in single reaction.

Results, discussion

Severe PPV2 and moderate PPV3 and PPV4 infections were detected. We detected positive signal of all examined viruses in lungs and lymph nodes in mononuclear cells in two basic

types: cells which have compatible morphology with lymphocytes and macrophages. Until now there are no available data about PPV2-4 cell tropism. Those results were in consistence with the nature of parvoviruses in general. In all cases in lungs PPV2-4 infected cells poorly express SLAIIDQ antigen and very rarely CD3 while lysozyme not at all: Most of the infected PPV4 cells did not express CD3 antigen, but there was small focuses where present PPV4 infected cells that expressed CD3 antigen were. Double staining SLAIIDQ/PPV4 failed to detect both molecules. In lymph node was obvious moderate B cell depletion, while examining serial cuts PPV4 positive cells were observed in area where both B and T lymphocyte were present. Since we rejected T cells it suggest conclusion that PPV4 infected cells are B lymphocytes. PPV2 was involved in presence of severe bleedings in lymph nodes in a background of PCV2 infection but more influence this virus it seems to have in lung pathology. PPV2 was involved in severe reduction of alveolar spaces and alveolar walls proliferation, in lungs will be much more alveolar macrophages, specifically in alveolar walls and T lymphocytes as a per bronchial inflammatory reaction in background of PCV2 infection. Lungs that was positive by *in situ* PCR for PPV2 presence where PCV2 negative while similar pathological condition was observed. In those PPV2 *in situ* PCR positive samples no pathogen could be related with this pneumonia. Proliferation of alveolar walls and reduction of alveolar spaces is typical lesion which can be attributed to viral infection like PCV2, PRRSV or SIV even classical swine fever will cause similar lesion in lungs. In a case of strong inflammatory reaction of T cells around bronchi, we can made relationship with *Mycoplasma hyopneumonia*. More likely this pathogen was not involved, necrosis of ciliary epithelial cells was evident in all case but IHC didn't detect it antigen in targeted cells. Since observed pneumonia can't be attributed to any causative agents and in the same time a strong infection of PPV2 was detected in lung parenchyma it is strongly suggest the pathologic role of PPV2 in these lesions.

PPV3 was responsible for much weaker inflammatory response in alveoli and much more mucus in bronchus, increase level of B cells in lungs and specifically T cells around blood vessels.

While results of our study indicate that maybe PPV2 and PPV3 are involved in pathogenesis of pneumonia alone or in background of PCV2 infection last investigated virus PPV4 seems to have nothing with respiratory disease. Only a few PPV4 infected cells were observed by *in situ* PCR in lungs free in alveolar spaces while pathological lesions and inflammatory reaction was very weak. PPV4 had much strong relationship with focuses of necrosis in lymph nodes and it seems that an amount CD3 cell is high in lymph nodes. This was quite interesting since necrotic

lymphadenitis was linked to PCV2 infection since it was present in 2% of cases. Results of the one Spanish study suggested that lymphoid necrosis in PCV2 associated disease affected pigs may be related to hypertrophy and hyperplasia of high endothelial venues (HEVs). The mechanism underlying these changes in HEVs was not clearly defined, but necrotizing lymphadenitis in pigs with PCV2 associated disease may develop following vascular damage with thrombosis and subsequent follicular necrosis. According our results PPV4 was also not related with lymphocytic depletion like necrotizing lymphadenitis. In one positive lymph node was observed strong infection of PPV4 in background of PCV2 infection. Since there is no relation between PCV2 and necrosis in lymph nodes, this may indicate that PPV4 can possibly cause necrotizing lymphadenitis.

In conclusion, thanks to the support of OTKA PD, we assessed the prevalence of newly described porcine parvoviruses and we analysed the phylogenetic and molecular evolution of these viruses. We developed several testing methods to examine the pathologic role of PPVs. These PPVs are detected mostly in lymphoid cells and organs suggesting immunosuppression in affected animals. The PPV3 and PPV4 importance seems to lover than PPV2. According to our results, the PPV2 infection cause mostly respiratory diseases resulting economic losses. The PPV2 is the most frequently detected newly discovered porcine parvovirus worldwide in background of co-infections with other known pathogens, also suggesting the importance of this virus. Many more examinations are need, but it seems that in the near future will be necessary to develop an effective vaccine to prevent the losses. A growing number of researchers working on these PPVs, and thanks to OTKA PD funding we are in the first line!