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According to a 2016 survey, more than 25% of the industrial pig farms (163 of the investigated 600) were infected with PRRSV in Hungary. To mitigate the high economic losses caused by the virus in 2010, a national eradication program was implemented with the goal to eliminate PRRSV. The successful completion of the PRRSV eradication plan was impeded by the lack of an effective vaccine and detailed knowledge about the diversity and geographical distribution of the Hungarian PRRSV strains. Our main objective was to survey the genetic diversity of the Hungarian PRRSV population in order to determine their geographical distribution and to follow their spatio-temporal alterations during a five-year period.

Investigation of the evolution of PRRSV based on ORF5 sequences

First a survey was conducted in 72 industrial pig farms with our participation in 2016. We determined the occurrence and diversity of the virus, and assessed the epidemiological status and phylogenetic relationships of the Hungarian PRRSV isolates. Overall, 2141 blood samples from 72 Hungarian herds were collected and investigated. Most of the farms (68) were represented by 30 blood samples. From 3 farms 24 blood samples were collected while one herd was represented by 24 blood samples. Individual blood samples were centrifuged, clarified sera was pooled, and these combined samples were used for viral RNA extraction. The presence of viral RNA was detected with an ORF7-specific reverse transcriptase (RT) PCR method by using OneStep RT-PCR Kit (Qiagen). The following primers were applied in the reactions: diagF (GAATGGCCAGCCAGTCAATC) and diagR (TCGCCCTAATTGAATAGGTGACT). 46 of the 72 pig herds proved to be positive for PRRSV, although the classical signs of PRRSV infection have been reported only from 10 of these herds. For phylogenetic comparison ORF5 sequences were amplified from the positive samples. In the first round of OneStep RT-PCR, ORF5EU-uniF (CAATGAGGTGGGCYACAACC) and ORF5EU_uni_R2 (GGGCAGGGGCCAGAATGTAT) primers were used to obtain a 982 bp long fragment. However, this approach resulted in sequenceable bands only in 15% of the cases. In the majority of the samples another round of PCR were needed to amplify a 600 bp long product with a nested set of primers (ORF5F-inner (ATGAGATGTTCTCACAAATTGGGGCG) and the ORF5EU-uniRr (TCGTCTAGGCCTCCCATTG)). This method led to success in around 50% of the samples. To sensitize RT-PCR and gain additional data from the remaining samples, cDNA synthesis were performed by SuperScript IV Reverse Transcriptase (Thermo Fisher) and two or sometime three additional PCR rounds were executed on the transcribed cDNA using diagF-diagR and ORF5EU-uniF-ORF5EU_uni_R2 primers and Phire Hot Start II DNA Polymerase. At the end sixty ORF5 sequences were obtained from 38 positive farms, and two virus pools were sequenced successfully from 22 farms. PCR fragments were purified from agarose gel and sequenced by Sanger method using amplification primers. Sequences were deposited (with a release date 10.31.2018) into the NCBI nucleotide database. Mega6 software package was used for the phylogenetic analysis. Sequence comparison revealed that from the 22 checked, at least 11 farms were infected with two, relatively distant PRRSV strains. All samples proved to be PRRSV-1 (Betaarterivirus suid-1) and were grouped within European subtype 1. The majority of the sequences could be grouped in five clusters: at least four of them could be fit into established clads. One cluster of samples was similar to the Amervac vaccine, other clusters were similar to a PRRSV isolate from Spain from 2003 (Spain 28/2003) and a Belgian isolate from 2010 (08V194). The closest relative of the third cluster

is a Belgian isolate (08V204). The fourth cluster is similar to isolates from South Korea, while the closest relatives of the fifth cluster are isolates from the Czech Republic.
In the 2017-2018 period 42 PRRS ORF5 sequences were gathered from blood samples of PRRSV positive Hungarian farms, they were compared to the sequences of older Hungarian isolates and classified by a method applied by Balka et al. (2018) in a paper suggesting a novel classification system for PRRSV. Briefly: ORF5 nucleotide sequences gained in 2016-18 were aligned with the sequences of earlier Hungarian isolates and with reference sequences using ClustalW in Geneious 9.1.8. The classification of reference sequences originated from Model selection was performed in MEGA 10.0.1 (Kumar et al., 2018): a general time-reversible nucleotide substitution model with four categories of gamma-distributed rate heterogeneity, and a proportion of invariant sites (GTR + Γ4 + I) was used. Phylogenetic tree inference was conducted using MrBayes 3.2.6 (Ronquist and Huelsenbeck, 2003) with 1 million generations, during which trees were sampled every 250 generations, and the burn-in was set to 10%. According to this method, the 2016 samples were reclassified into 6 clades: 1A, 1D, 1F, 1G, 3D and 3F within Subtype 1. The 2017 samples fall in 1A, 1B, 1D, 1F, 1G, 3D and 3F, while the 2018 samples are grouped into 1A, 1D, 1E, 1G, 3D. We concluded that Hungarian strains were highly diverse, which implied multiple viral introduction events from surrounding Central European and Western European countries. However, diversity showed a decreasing tendency in time due to the decline of infected farms and the more stringent animal health rules for live animal import imposed during the execution of the eradication plan. The later identified strains were most probably the descendants of the viruses that have already been circulating in Hungary. It is highly probable that not even the newly identified more diverse (compared to the 2016 strains) 1B (2017) and 1E (2018) strains were "newly introduced viruses", because they were close relatives to earlier (2009-2015) identified Hungarian PRRSV strains which most probably remained undetected by us in 2016.
Interestingly, the analysis of the phylogenetic tree suggested that at least some lines belonging to clad 1E may have evolved significantly slower than the average PRRSV lines. In this clad several viruses isolated in 2013 and 2017 or 2009 and 2017 show minimal sequence diversity from each other in their ORF5 sequences. There was no sign of the appearance of dramatically different "non-Hungarian genotypes" (notably highly virulent East-European subtype 3 viruses, like the Lena strain (Karniychuk et al., 2010) during the investigated period.
In the 2018-19 and the 2019-20 period, the ORF5 sequences of 59 and 43 field strains were determined and compared to a diverse reference set of viruses. All isolates clustered into clades 1A, D, F and G, and furthermore into clades 3D and F within Subtype 1 of the species PRRSV-1. With the advance of the Hungarian PRRSV eradication plan, the number of PRRSV-infected farms significantly declined from ~61% (2015) to less than 5% in 2021. Therefore the number of isolated PRRSV field samples also decreased significantly. In 2021 we sequenced the ORF 5 gene of 20 PRRSV-1 isolates originated from 11 Hungarian farms. Sequence analysis revealed that most of them (12) belonged to viruses with close relations to vaccine strains authorized in Hungary. The remaining isolates could be classified as members of clad 3D subtype 1, and they were characteristic to a narrow geographical area. Unfortunately one pig farm was found to be serologically positive to PRRSV-2 (Betaarterivirus suid-2) and sequence analysis revealed the presence of a PRRSV-2 vaccine strain which is routinely used in Danish farms. Epidemiology investigation confirmed the Danish origin of the infection by the import of non-vaccinated but vaccine virus-infected animals. Due to extensive pig transport between Hungarian farms and insufficient biosecurity measures the virus spread to other eight farms in the area owned by different companies. (Zádori et al.,2018; Olasz[1] et al., 2019; Olasz[2] et al., 2019)

Balka et al. (2018) distinguished three lineages with 14 sublineages (1A-G, 2, 3A-G). During 2017-2019 we managed to sequence nearly 200 PRRSV isolates from Hungary and investigated their phylogenetic relationships with the method applied by Balka et al. (2018) (Bayesian Markov chain Monte Carlo method) selecting only a few reference strains for every sublineage. However, the resulting phylogenetic tree did not support the original structure established by Balka et al. (2018). When the phylogenic relationships of ~200 novel isolates and reference strains from major branches were investigated, several anomalies were found, including the disappearance of lineage two, the formation of uncharacterized, newly segregated branches, and changing the relative position of the reference strains. All of these findings significantly altered the structure of the original tree. These results prompted us to create a classification scheme that is suitable for building a stable universal tree, which is able to integrate the newly emerging PRRSV variants without the necessity of continuously reorganizing the system. We took all available PRRSV ORF5 sequences from Genebank and tried to build a tree with Bayesian approach applying the sampling parameters used by Balka et al. (2018). However, even after increasing the number of samplings to 2 millions, a consensus tree could not be reached with this method, verifying that the Bayesian approach is not suitable for the classification of large numbers of relatively short sequences (Kaján personal communication, 2018). Since such sequences can be best classified with maximum likelihood (ML) method, we applied this technique to get a scientifically more reliable tree, unfortunately unsuccessfully. Based on the bootstrap values we could distinguish only four well defined clads (clad I-IV) with an uneven distribution of the investigated strains, clad I containing 95% of all sequences. Other anomalies aroused using ML method: clashes with Balka et al.'s classification, such as translocation of the major clads, appearance of non-monophyletic lineages and unclassified new branches. However, the biggest problem was that we were unable to establish unambiguous clad demarcation criteria among the major clads (clad I-IV) and inside clad I based on sequence identity. Therefore we had to conclude that the partial ORF5-based classification of PRRSV-1 strains became obsolete and this relatively short sequence stretch (~430 nucleotide) of this highly variable RNA virus is not suitable anymore to classify the ever increasing numbers of PRRSV isolates.

Continuing our study we tried to solve this problem finding a more appropriate region of the PRRSV genome for phylogenetic classification. Therefore we extracted the available ~170 (near) complete PRRSV-1 genome sequences from Genbank. After comparing different regions of the genome (full length, complete ORF5, ORF1B amino acid, ORF1B nucleotide) we reconstructed the corresponding phylogenetic trees. In all cases the three subtypes originally described by Stadejek et al. (2006) could be distinguished, and their separation was confirmed by high bootstrap values. However, we were again unable to establish clear clad demarcation criteria based on sequence identity (in each tree, several interclad sequence identity values were higher than some of the intraclad sequence identity values) even among the three subtypes, not to mention intra-subtype lineages. We suspect that besides high mutagenicity, frequent recombination among different PRRSV lineages could be blamed for this anomaly (Kaján et al., 2021).

Since computationally extensive "traditional" algorithms (Bayesian or ML) were not able to supply scientifically reliable classification scheme, we also used a minimum spanning similarity network application for investigating the relations among PRRSV strains. This approach revealed that ~37 % of the Hungarian PRRSV strains, mostly identified during the 2015-2019 period, are most probably vaccine-originated. Among the vaccine strains 74.3%, 21.5% and 4.2% were found to be Porcilis, Unistrain (formerly Amervac), and Reprocyc originated,respectively. The majority

(96%) of Porcilis-related strains showed 100% homology or very high sequence identity (99.1–100%) in the ORF5 region. The Unistrain and the Reprocyc-derived sequences were somewhat less stable in their ORF5 sequences (95 and 91% showed 98-99.9% similarity, but hardly a few showed full sequence identity). This data suggest that Porcilis vaccine can be the genetically most stable vaccine in the field, while Unistrain and Reprocyc strains are more inclined to change at least in their ORF5 region (Bálint et al., 2021).

In vitro evolution studies

We have generated the full-length clones of a mildly virulent Hungarian (Hu9625/2012) and a highly virulent subtype 3 virus (strain Lena) under the regulation of the CMV promoter to genetically manipulate and study the effect of the exchange of RNA-dependent RNA polymerase (RdRp) of the virus. Despite that sequencing of the clones did not reveal any significant differences to the sequence of their parental genomes, even after several attempts we were not able to rescue viruses from these clones neither in MARC-145 cells, nor in BHK-21 cells. So we decided to circumvent the problem by measuring the mutation rate of the PRRSV SD01-08 strain (that can be rescued from its clone in MARK-145 cells) and exchange its RdRp fragment to that of the strain Lena and Hu9625/2012. However, the fragment change did not result in rescuable viruses.

We investigated the stability and evolution of the PRRSV NSP2 in porcine alveolar macrophage (PAM) cells. Nsp2 is one of the most variable regions (hypervariable) of the PRRSV genome. In many very closely related PRRSV virus isolates the similarity index at the NSP2 sequence falls below 0.5. We theorized that this region is more sensitive for mutation than other parts of the genome and a pool of mutants is generated (quasispecies) in PAM cells (the primary target cells of the virus). From this quasispecies pool a dominant one (master sequence) would arise by selection or by simple genetic drift in different organs of the animals. In order to prove our theory, we sequenced the plaque-purified virus Hu2375 grown in PAM cells by ultra-deep sequencing (>15 million read) expecting a large amount of single nucleotide variance (SNV) in this region. 31 SNVs (minimum variant frequency 0.1, average coverage 78 000) were detected in the viral coding region, among them 5 in the NSP2 (16% of the variants). Since NSP2 covers ~22% of the coding region (3234/14772 nucleotide) we concluded that no outstanding variance could be detected in the NSP2 region with this *in vitro* approach (growing PRRSV in PAM cells), which is contradictory to the *in vivo* observed phenomena (rapid quasispecies evolution and increased variance in the NSP2 during early infection). These findings strongly suggest that *in vitro* PAM infection cannot be used as model for PRRSV evolution.

We also analyzed the function of a short alternative open reading frame named ORF7a that was discovered by us within the ORF7 region of the PRRSV genome. Short proteins (7ap) translated from the ORF7a of PRRSV-1 and PRRSV-2 are able to completely abolish the motility of nucleic acids at relatively high molar charge ratios in gel retardation assays indicating strong dsDNA- and ssRNA-binding capability. Conserved RNA- and DNA-binding properties suggest that nucleic acid binding is a functional property of the divergent 7aps, and it is not an arbitrary consequence of their net positive charge. Sera from 7ap-immunised pigs and mice did not react with 7ap or 7ap-GFP; however, antinuclear antibodies were detected in the sera of the immunized animals, suggesting an ability of 7ap to interact with or mimic autoantigenic macromolecules. It seems that after translation, 7ap is first transported into the nucleus, and later it also accumulates in the cytoplasm (Olasz et al., 2017) Another suspected nucleic acid binding short viral protein (3ap of

feline enteric coronavirus (FECV)) with similar multiple (cytosolic and nuclear) localizations was also identified by us in another nidovirus species (FECV) (Mészáros et al., 2018).

Whole genome sequencing and sequence analysis

The difficulties of whole genome sequencing from field samples, and as a consequence, the lack of data are some of the main reasons why researchers have limited understanding of PRRSV evolution in the field. We have made progress in developing a simple universal method to sequence PRRSV isolates from stored field samples. Field samples contain different amounts of PRRSV RNA, usually between Ct 17-35 determined by diagnostic RT-PCR. Yet, most of the time we were not able to gain full sequences even from samples originally determined to have high PRRSV content and low CT values. Therefore we systematically analyzed the possible reasons of this failure. Quantitative PCR analysis proved that a combined RNAse-DNAse treatment of blood samples is able to reduce the genomic DNA background to a very low level (below Ct 35-40 measured by 16s rRNA gene PCR) after centrifugal clarification and limited sample dilution. This background reduction was sufficient to allow the new generation Illumina sequencing of the PRRSV genome after reverse transcription and whole genome amplification by Phi29 DNA polymerase. Interestingly, following reverse transcription, the Ct of the genomic background decreased with the usual 1-2 values, suggesting that the samples still contain some nuclease resistant RNA, most probably associated to proteins. Probing freshly PRRSV-spiked samples revealed that genome concentration around 25 Ct/ul in the samples could be sufficient for successful whole genome PRRSV sequencing if DNA concentration of the genomic background remains below detectable levels (Ct<40). This finding suggested that inappropriate handling, transport and storage of PRRSV samples can be the main reason of whole genome sequencing failures. In fact, very high Ct increase (>10 Ct) was found in all PRRS field samples stored in -20 ℃ after thawing, compared to their original value measured before freezing, indicating that only fresh or -70 ℃-stored PRRSV samples are appropriate for whole genome sequencing. Our approach can be generalized and applied to the whole genome sequencing of any animal viruses from bodily or tissue culture fluids (Olasz[3] et al., 2019).
We managed to analyze the full nucleotide sequence of 10 isolated PRRSV strains.
Twelve viruses from different farms were isolated on porcine alveolar macrophage cells in 2016. For the full genome sequencing of six isolates, RNA was extracted with Zymo Direct-Zol Miniprep plus kit and RiboZol RNA extraction Reagent. The DNA library was prepared using the NEBNext® Fast DNA Fragmentation & Library Prep Set for Ion Torrent with the Ion Torrent Xpress barcode adapters. Libraries were sequenced with an Ion Torrent PGM sequencer. The complete nucleotide sequences of the six viruses were deposited into the NCBI nucleotide database (HUN60077, HU24924, HU19483, HU19401 HU18861 HU18755; their Accesssion numbers, respectively: MK167464.1 MH463459.1 MH463458.1 MH463457.1 MH463456.1 MH463455.1). A phylogenetic analysis was conducted to classify our Hungarian isolates with complete nucleotide sequences together with all complete PRRSV genome sequences available in the Genbank. Interestingly, the six isolates showed unexpectedly high divergence from other PRRSV strains and from each other as well. The two most divergent strains were HU18875 and HU19401 showing 19% nucleotide difference at full genome level. The 3F clade representative HU18875 and HU18861, that were isolated from the same town but not from the same farm, proved to be the most similar to each other with around 9.5% difference. However, sequence similarity was not uniformly distributed on the genome: a highly different region was detected between nucleotide

2000 and 5500 in the nsp2-4 region which suggested recombination. Simplot analysis confirmed this idea, though potential recombination partner could not be identified from data banks. Hu19401 may also contain a recombinant part between nucleotide 7275 and 9328 in the nsp9-RdRp region showing very high similarity to that of the Lelystadt virus. As for potential recombination origin, HUN60077 supplied the most convincing evidence. Eight major ORFs were compared individually to PRRSV sequences available in the GenBank database. The genomic regions encoding ORF1b and ORF3 to ORF7 were highly similar to the corresponding genomic regions of the vaccine strain Unistrain (MK134483.1). In contrast, the genomic regions coding ORF1a and ORF2 were not closely related to Unistrain; additionally, no highly similar sequences from within this genomic region were identified in GenBank. Phylogenetic analysis of different genomic regions was consistent with the possibility that strain HUN60077 could have evolved through recombination between an Unistrain-derived strain and another, yet unidentified, PRRSV strain. To confirm this hypothesis, the algorithms RDP37, GENECONV38, MaxChi39, Bootscan/Recscan39, SiScan40, and 3Seq41 were utilized implemented in the software package RDP4 (Martin et al., 2015). Furthermore, a sliding-window analysis tool, SimPlot (Lole et al., 1999), was applied to visualize the putative recombination breakpoints. In this analysis, ~80 nearly complete genome sequences of PRRSV-1 were downloaded from GenBank and Unistrain was identified as a likely parental strain, providing large fragments of ORF1b and the 3' end region of the genomic RNA including the ORF3 to ORF7 region. However, we were unable to identify the other parental strain that donated ORF1a and the genomic region encompassing the 3' end of ORF1b and the whole ORF2. Although the putative parental strain donating these protein-coding regions could not be determined by this approach using the available limited data set, our findings of a probable recombination event were confirmed using seven recombination detection algorithms, as well as Simplot analysis. It was the second occasion that recombination of a PRRSV vaccine strain with a field strain was documented.

The analysis of the six strains also revealed that they have highly divergent NSP2 regions that differ from each other and from any known isolates. According to Simplot (Lole et al., 1999) analysis it seems to be very likely that one of the isolates that showed moderate virulence evolved by multiple recombinant events between a vaccine strain-related virus and an unknown ancestor. The coding regions of NSP9 (RNA-dependent RNA polymerase), NSP10 (helicase), NSP12, E, GP3, GP4, GP5, M and N proteins seem to be inherited from the vaccine strain-related virus.

We also determined the full nucleotide sequences of four additional isolates (HU11933, HU17537, Hu2371 and Hu2375). The nucleotide sequence difference between HU11933 and HU17537 was 13%. The full-length sequence of PRRSV HU11933 was 15,093 nt long. At the genome level its closest relative proved to be PRRSV strain 14432/2011 (KR296711) isolated in 2011 in Hungary, with the overall nucleotide identity 88%. A 14,941-base-long consensus sequence was obtained from HU17537. Its closest relative strain proved to be the PRRSV MLV-DV (with a nucleotide sequence difference 5.88 %) suggesting a vaccine origin for this isolate.

The genome sequence (15,082 bp) of strain Hu2371 showed the highest whole-genome sequence similarity to another vaccine strain, the Amervac PRRS (95.79% identity). Simplot analysis suggests that Hu2371, a recombinant virus originated from the recombination of an Amervac-derived strain and another, yet unidentified, PRRSV strain, swapping a ~2000 bp fragment at the end of the 1b ORF that includes the RNA-dependent RNA polymerase.

Hu2375 was found to be 14,932 nt in length. The phylogenetic analysis of the whole genome revealed that its closest relative is the PRRSV strain AUT14-44 (KT334375.1). The nucleotide identity among them is 92.70%. Analysis of the full genome sequences supports and supply

additional evidence to the conclusion of our ORF5 investigations that large proportion of the circulating PRRSV strains in Hungary are most probably vaccine-derived and recombination between field and vaccine strains played a major role in their evolution (Marton et al., 2019; Olasz[3] et al., 2019).

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