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Sequence analysis of Hungarian PRRSV isolates and the immunological, biochemical characterization of 7ap

Theses of PhD dissertation

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1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) was first detected in the USA in the late 1980s and the new disease was characterized by reproductive and respiratory symptoms, reductions in growth performance and increased mortality in young pigs. The pathogen was identified in 1992 and the first isolate was named VR-2332. A similar illness and virus was described in Europe a few years later. The later studies have shown that European and North American strains are genetically related.

The PRRS spread all over the world and caused serious economic losses in the swine industry. Two types of commercial vaccines are available against the PRRSV infection: modified-live virus (MLV) vaccine and killed virus (KV) vaccine. Despite the effort of research laboratories and vaccine producer, the vaccines provide only partial protection against PRRSV infection.

The development of effective vaccine is prevented by several factors. One major problem is high degree of genetic diversity among the two genotypes of the virus; it results in extreme antigenic variation of the genomes. Two virus isolates can exhibit up to 45 % variability in

nucleotide sequences. Another problem is the localization and the function of the genomic regions, which affect the virulence and pathogenicity. These factors, are not fully understood. The PRRSV infection induces a weak immune response in the animals as a consequence of its various immunosuppressive proteins.

PRRSV was first identified in Hungary in the middle 1980s, but the prevalence only started to rise after 2000s. A heterogeneous PRRSV population developed in Hungary over the years, which is genetically more diverse than the population in Western Europe. Strains of European and North American genotypes can be found among a Hungarian PRRSV isolates. Because of the serious economic losses, a National PRRS Eradication Program was approved in 2010 in Hungary.

2. Aims

- It was assumed the two Hungarian PRRSV isolates possess specific genetic characteristics. To determine the full-length genome sequences of these two PRRSV isolates.
- To identify new, previously unknown ORFs in the rapidly evolving PRRSV genomes.
- To prove protein expression from alternative ORFs during the viral infection and characterize the biological properties and functions of these proteins.
- To investigate the antigenicity of the hypothetical 7ap protein using the sera of immunized or PRRSV infected pigs. To identify the localization of 7ap in the cells and to study its biological properties.

3. Methods and material

3.1 Origin of isolates, analysis of nucleotide sequence and reconstruction of the phylogenetic trees

One of isolate (9625/2012) was originated from an epidemiologically closed, non-vaccinated, originally PRRS-free fattening herd in Füzesgyarmat (Békés country) during 2012, another isolate (PRRSV-2/Hungary/102/2012 (102HU)) was obtained from the young growing pig originating from an endemically PRRS positive herd in Ács (Komárom-Esztergom country). The PRRSV was isolated from the lymph node and the lung. The tissues were homogenized and porcine alveolar macrophages (PAMs) were inoculated with 100 µl of supernatant. The RNA was isolated with QIAamp Viral RNA Mini Kit and cDNA was generated using Superscript III First-Strand Synthesis System kit and a T_{20} primer.

The genome of PRRSV 9625/2012 was amplified in five or six overlapping parts. The fragments were determined using the next generation sequencing. The nucleotide sequences were assembled and aligned with the SeqMan Ngen software. The phylogenetic

reconstruction based on full-length genome and ORF5 gene and the alignment of DNA and protein sequences were performed with MEGA software. The evolutionary history of the 102HU was inferred using the maximum likelihood method based on the Tamura–Nei model and the phylogenetic tree of 9625/2012 was reconstructed with the neighbor-joining algorithm.

3.2 Research methods of ORF7a

To identify unrecognized ORFs with potential translational products, sequence analysis was performed with the ORF finder tool on sequentially divergent PRRSV genomes. The conservation the hypothetical ORS were examined with alignment of the translated protein sequences. The three alternative frames of the nucleocapsid gene (ORF7) were fused to eGFP in the pEGFP-N1 vector. In each construct, approximately two third of the nucleocapsid gene was cloned from the TRS sequence to the last codon of the hypothetical ORF into vector. The PCR products were digested with *Xho*I and *Bam*HI and they were ligated into the same restriction sites of the vector. To create 3xFLAG constructs, first the

pcDNA-FLAG vector was created by cloning the 3xFLAG into pcDNA3.

The same protocols and enzymes were used to create the Flag fusion constructs as the eGFP fusion systems. All constructs were transfected into PT cells with Turbofect and the cells were fixed with formaldehyde solution after 48h. The presence of FLAG fusion proteins was detected by indirect immune fluorescence using the mouse anti-FLAG M2 monoclonal antibody as the primary antibody. The samples were examined under a Zeiss inverse fluorescence research microscope.

ORF7a peptid from two different strains were synthesized by CASLO ApS. The ELISA plate was coated with 7ap solution, and then different HRP-conjugated antibodies were serially diluted with blocking buffer and added to the wells. Then ABTS solution was added and the optical densities (ODs) were measured with an ELISA reader.

To set up the complement fixation system freshly prepared sheep erythrocytes was mixed with diluted hemolytic amboceptor (rabbit anti sheep erythrocytes IgG) and serially diluted (4-0.4% in ten steps) guinea pig complement. In the inhibition test, the lowest

complement dilution where full lysis (2%) occurred was chosen, and to exclude false positive results, one percent higher concentration complement was applied. All tested components including Hu7ap, Wu7ap and spA were investigated in twofold serial dilution.

The interactions among the 7ap peptides and different type of nucleic acids were tested in the DNA and RNA gel retardation assay. The RNA probe was transcripted from a Flag-fusion construct. The double stranded DNA sample was prepared from a plasmid by digestion with PvuII and HindIII. The PRRS3END oligonucleotide primer was used as the single stranded DNA probe. The Wu7ap and the Hu7ap peptides were added in increasing concentration to the nucleic acids (to 1.3 µg RNA, 0.5 µg double stranded DNA, 200 ng single-stranded DNA). The samples were loaded onto a 6% non-denaturing polyacrylamide gel casted by the Ornstein-Davis method and run at 110V in Tris-Glycine running buffer. Nucleic acids were visualized with GelRed Nucleic Acid Gel Stain.

The interactions among antibodies and 7ap proteins were investigated in protein-protein gel retardation assay. The 7aps was added in increasing concentration to 8-10 µg IgG (to mouse monoclonal IgG

and pig polyclonal IgG) or IgG fragments (IgG (Fab') and IgG Fc fragment).The final volume was adjusted to 14 µl, and the samples were analyzed by electrophoresis at 120 V for 2 hours on a clear native polyacrylamide gel. The gel was stained with 0.1% Coomassie Brillant Blue R250.

Two BALB/c mice were immunized intramuscularly with a double dose of 7ap contained emulsion. The two injections were separated by a two-week interval. The K1p injected mice served as negative controls in this experiment. Two 6-week-old pigs were obtained from a PRRSV free herd. The pigs were immunized intramuscularly with a double dose of 7ap contained emulsion. The two injections were separated by an interval of 17 days. All animals were sacrificed 3 weeks after the second injection.

The antinuclear antibodies (ANA) were detected with indirect immunofluorescence. The diluted mouse and pig sera from the immunized animals were used as the primary antibodies. The secondary antibodies were the goat anti-mouse and goat anti-swine IgGs in 1000-fold dilution.

4. Result

4.1 Full genome sequencing of Hungarian PRRSV samples (9625/2012 and PRRSV-2/Hungary/102/2012 isolates)

The identity of nucleotide sequence of 9625/2012 isolate was 96% with PRRSV strains Amervac MLV and 95% with PRRSV strains Olot/1991. Alignments to the closest relatives revealed no deletion or insertion in the genome. SimPlot analysis was performed with all the full-length PRRSV genomes but no sign of recombination was observed. The phylogenetic tree using ORF5 sequences revealed that PRRSV 9625/2012 is a Type 1, subtype 1, Clade D virus.

The amino acid (aa) sequence of the 9625/2012 was aligned to Amervac MLV and Olot /1991. In the two neutralizing epitopes of GP2, two and one aa substitutions were found. In the non-neutralizing ARs, two-two aa substitutions were observed, respectively. In the non-antigenic part of the protein, only three aa differences were found.

In the two neutralizing epitopes of GP3, two and three aa substitutions were found, respectively, whilst in the non-neutralizing AR II contained two mutations.

Four aa substitutions were found in the neutralizing epitope of GP4, while three mutations were observed in the non-immunogenic part of the protein.

In the neutralizing epitope of Type I PRRSV GP5, one aa substitution was found. Four other epitopes were observed on the protein, two of which were conservative and the other two contain one-one aa substitution. Large proportion of amino acid changes were found in the antigen regions of GP2, GP3 and GP4, suggesting the selection pressure of the immune system could have played an important role in the mutation of the viral sequence.

The phylogenetic analysis of the whole genome of PRRSV-2/Hungary/102/2012 (102HU) showed that it was a novel type 2 PRRSV isolate that was phylogenetically close to the progenitor type 2 PRRSV, and not related to VR2332 or Ingelvac PRRSV MLV. Comparisons of the full genome showed that even the most similar strain, VR-2385, was only 87% identical. The 102HU on the ORF5 dendrogram was clustered in lineage 2 PRRSV. The 11 most similar sequences were 8–92% different. The RAT analysis showed no evidence of recombination anywhere in the genomes of all whole genome sequences available to us.

102HU has deletions of 10 amino acids and 9 amino acids in the nsp2 that were not present in prototype strain VR2332 or the prototype high pathogenicity Chinese strain JXA1. By contrast, there was a 9 aa insertion in the nsp2 (102HU aa 795–803) that was not present in VR2332 or JXA1.

The antigenic regions (ARs) and glycosylation sites within glycoproteins of 102HU have been analyzed and compared to references strains. Although majority of mutations were not on the epitopes of GP2 and GP3, but their localization was not random. The mutations were found on the signal peptide and C-terminal end of GP3 after the TM region and because of overlapping, on the N-terminal end of GP4. The $AR_{51–65}$ is a hypervariable region of GP4; five aa substitutions were detected compared to the reference strains. The two T-cell epitopes contained two and three aa substitutions compared to the other strains. While four N-glycosylation sites in this protein were recognized in the reference strains, an additional, fifth N-glycosylation site was observed within AR51–65 in GP4. The presence of at least six B cell epitopes was reported within GP5. Two ARs ($AR_{1–15}$ and $AR_{27–35}$) at the N-terminus of the protein are very variable while the other four epitopes are

conserved. The three T-cell epitopes of GP5 do not contain aa substitutions in 201HU compared to the reference strains. GP5 contains five potential N-glycosylation site, two sites (N44 and N51) are highly conserved and were found within $AR_{37–51}$. The other three glycosylation sites (N30, N34 and N35) were within the heterogeneous $AR_{27–35}$.

4.2 Immunological and biochemical characterization of 7ap

Sequence analysis revealed a short alternative ORF named ORF7a within the nucleocapsid gene. The hypothetical translated protein was named 7ap, and its length was between 26 and 53 aa depending on genotypes continued in a coding region stretching.

The most plausible explanation of the universal presence of ORF7a and the observed conservations of the coded proteins in sequence and physicochemical characteristics is that ORF7a encodes a protein. Three constructs were created from the ORF7a gene: the N protein was fused to the eGFP reading frame as a positive control. The eGFP reporter gene was joined to the reading frame without ORF as a negative control.

The reading frame encoding the hypothetical 7ap was fused to the eGFP protein in the third construct. The positive control construct encoded the nucleocapside-eGFP fusion protein resulted in a robust fluorescent signal in the cells. Strong fluorescent signal was detected, although with lower intensity, in the case of 7ap-eGFP fusion protein construct. FLAG expression could be detected in cells transfected by the frame +1 and frame +2 constructs. These finding were verifying that 7ap is in fact translated *in vitro* from the nucleocapsid mRNA. Soon (16–20 h) after transfection, the majority of the fluorescent signal appeared in the nucleus, while later (48 h) it could be detected in the whole cell.

If the translated 7ap has immunogenic properties, antibodies may be produced against the 7ap in the swine. Surprisingly, strong HRP signals were detected in all Hu7ap-coated wells, regardless of the presence or absence of primary sera (including the one from a non-infected animal). This finding suggested an ‘aspecific’ antigen-binding region, independent of the IgG binding capability of 7ap. The experiments revealed that Hu7ap binds strongly to all investigated mammalian (pig, mouse, goat, and rabbit) IgG horseradish peroxidase

(HRP) conjugates and that the binding is independent of HRP.

Protein-protein gel retardation assay was performed with Hu7ap to confirm the results of ELISA and to localize the binding site/s of 7ap on swine IgG. Hu7ap and Wu7ap were able to completely inhibit the entry of the monoclonal mouse IgG and polyclonal pig IgG. Similarly, applying in increasing concentrations of Hu7ap and Wu7ap titrated out completely polyclonal pig IgG(Fc) fragment, while only a small fraction of the pig polyclonal IgG$(Fab)_2$ was blocked running into the gel even when Hu7ap and Wu7ap were applied in large excess. These experiments verified that despite their sequence heterogeneity, both 7aps are able to bind both pig and mouse IgGs and the main binding site of swine IgG is localized on the Fc fragment.

The complement fixation inhibition test suggested that Hu7ap binds to the CH2 domain of the Fc, and its binding site at least partially overlaps with the C1q binding site.

7aps contain several arginines and have a net positive charge that is the hallmark of nucleic acid binding proteins. Nucleic acid-7ap a gel retardation assay was performed to verify this hypothesis. The result

indicated both 7aps have the highest affinity to dsDNA while they bind ssDNA the least.

To further investigate the role of 7ap in the life cycle of PPRSV we intended to raise antibodies against Hu7ap. Two mice and two pigs were immunized with 7ap containing the vaccine. No significant differences were detected against Hu7ap in ELISA between the sera of Hu7ap immunized animals and those of non-immunized animals. However, antinuclear antibodies (ANA) were detected in the sera of the animals immunized with Hu7ap. Interestingly, the sera of the two animal species reacted differently with the nucleus. The sera of pigs showed a distinct fine-speckled staining with resting cells, and did not react with the chromosomes of dividing cells, whereas mouse sera reacted strongly with the chromosomes of mitotic cells indicating the presence of dsDNA-binding antibodies.

5. Discussion

5.1 Characterization of PRRSV 9625/2012 strain

The difference between the two PRRSV serotypes can be 30-45 % in the nucleotide sequences. A conservative estimations put the mutation rate

somewhere between 1.8 and 7×10−3/nucleotide/year (27, 28), which still places PRRSV among the most rapidly evolving viruses known. The 4% difference observed between 9625/2012 and Olot/1991/Amervac MLV may equate to 4-8 years of changing in field. PRRSV 9625/2012 had higher genetic distance from the vaccine strain. The most plausible explanation of the origin of PRRSV 9625/2012 is that it descended from Amervac MLV or an Olot/1991-like, unidentified PRRSV between 2004 and 2008, and it circulated without identification in Hungary until its detection in 2012. Analysis of the putative amino acid sequence revealed the presence of relatively high (9% and 7%) aa differences in Nsp1 and Nsp2 between 9625/2012 and related viruses. This phenomenon may correspond to the fact that these proteins are involved in the blockage of the type I interferon synthesis and signaling pathway. The ARs within GP2, GP3, GP4 and GP5 proteins of PRRSV 9625/2012, the main targets for neutralizing antibodies were compared with those of Amervac MLV and Olot/1991.

The two aa substitutions in the neutralizing epitopes of GP2 and the three aa differences in the non-neutralizing ARs, versus the five aa substitutions in the

non-antigenic part of the protein suggest the role of selective immune pressure in the development of this strain.

Six mutations from the 16 can be found the antigenetic region of the GP3 protein, so the mutation was produced by the selective immune pressure. In GP4, the three aa substitutions were found within a neutralizing epitope confirm that this region is under antibody-mediated pressure in vitro and in vivo. The three amino-acid substitutions present from aa 36-38 spanning the two N-terminal epitopes of GP5 may suggest the role of selective immunological pressure.

A plausible explanation for the severe clinical signs and pathological lesions observed during the outbreak could be the presence of a concurrent Mycoplasma hyopneumoniae infection. Previous studies have shown an increase in the severity of the pneumonia induced by dual, compared to single infection either with Mycoplasma hyopneumoniae and PRRSV.

5.2 Characterization of 102HU strain

Type 2 PRRSV strains are predominant in North America and Asia. 102HU is the first European, wild, type 2 isolate, which is clearly not related to Ingelvac

PRRS MLV. Moreover, the genome was only 87% identical to the most similar genome available in GenBank proving its unique status. The phylogenetic analyses performed with the whole genome. The phylogenetic analyses revealed that it is a member of an ancient lineage 1 or lineage 2 clusters whose earliest sequence was reported from Eastern Canada in the early 1990s

The Eastern Canadian origin of both lineages 1 and 2 suggested that our Hungarian strain was introduced in Eastern Europe within the past 10–15 years.

A similar strain was isolated from Slovakia close to the Hungarian border and the proximity of the border to the isolation site of 102HU makes it probable its ancestor strain came from Slovakia, but the other direction is also possible. The sequence homology between Slovak and Hungarian isolates and the significant difference from all other European sequences confirms that assumption they originate from a common source.

Antigenic regions of GP2 are relatively conserved, so it is likely that they are functionally important and PRRSV does not tolerate amino acid changes in these regions. The majority of amino acid changes among are positioned in the N-terminal (GP21–

40) and the C-terminal (GP2240–256) regions. Excluding AR137–159, the predicted B cell epitopes of GP3 are conserved. The reasons for conservation might be similar to those of GP2.

The conserved C terminal anchor overlaps with the variable signal peptid. Despite the great variation of sequence of signal peptide the function remains unchanged. The reason is the amino acid composition can vary widely. The sequence of the transmembrane (TM) region could also be theoretically variable; TM helix function (helix with hydrophobic amino acids) does not explain the conservation. This sequence pattern suggests that the amino acids in membrane anchors of GP2 and GP3 have additional sequence specific functions (e.g. protein–protein interaction).

$AR_{51–65}$ is a hypervariable region in GP4, is considered as neutralization epitope in type 1 PRRSV virus, it was demonstrated that this epitope is susceptible to monoclonal antibody-induced immunoselection in vitro. The AR51–65 contains a fifth N-glycosylation site (N57). It can be speculated that this potential glycosylation site might function as an anchor point for glycan shielding.

$AR_{27–35}$ within GP5 may function as a decoy epitope,which is hypervariable and is not involved in neutralization. It was demonstrated that aa positions at 32, 33 and 34 of GP5 are under significant positive selection. N30, N34 and N35, together with the highly conserved N44 and N51 compose a very rare combination of N-glycosylation sites on the GP5 of type 2 PRRSVs, less than 1% of the strains contain this pattern. As a large majority of the amino acid alterations in GP4 and GP5 of our isolate were found on antigenic regions, we hypothesize that immunological pressure played an important role in the evolution of the virus.

5.3 Characterization of 7ap peptid

Despite the rapidly changing codon sequences of the main ORFs, recently several positionally conserved alternative ORFs have been identified in the PRRSV genome: ORF5a and ORF2b overlapping with the ORF5 and ORF2a.

Study of GFP and FLAG fusion constructs of the ORF7 region verified the translation of ORF7a. The conserved position of a methionine at the beginning of ORF7a in different PRRSV strains suggests start codon initiation of

the ORF7ap in all PRRSV strains. The translation of alternative frames in the nucleocapsid gene of several other nidoviruses (SARSV, bovine coronavirus 1 and mouse hepatitis virus (MHV)) belonging to the genus Betacoronavirus was reported previously.

The remarkable biochemical characteristics of PRRSV 7ap suggest an immunosuppressive function as well. 7ap binds to the Fc part of mammalian IgGs and inhibits complement activation. . In the classical pathway of complement activation the triggering event is the binding of the globular head of the C1q to the CH2 domain of IgG. Considering its strong positive charge and the high number of arginine residues in 7ap, it is tempting to speculate that 7ap binds to aa of the CH2 domain, contributing to the Fc C1q interface, and this binding inhibits Fc C1q interaction and complement fixation.

Several viral proteins have been identified in members of different virus families (Poxviridae, Retroviridae, Herpesviridae) that inhibit virus neutralization by interaction with the components of the complement pathway. Binding of Fc receptor-like viral proteins to the Fc domain of IgG with immunosuppressive effects was also reported from several viral species (HCV, HHV-5, MCMV). However, to our knowledge PRRSV 7ap is the

first reported viral protein which binds to the Fc domain of IgG and inhibits complement activation.

The experiments here proved that in addition to its IgG binding capability, 7ap also has the potential to interact with nucleic acids. Several highly positively charged peptides and proteins are unable to bind to nucleic acids, yet the two investigated 7aps relatively strongly bind to dsDNA and ssRNA. High sequential divergence and conserved RNA- and DNA-binding properties make it highly plausible that nucleic acid binding is functional, and it is not just an arbitrary consequence of the net positive charge of 7aps.

The localization suggests multiple functions for the protein. One of the functions in the nucleus might be the perturbation of the normal host mRNA transcription and processing. The cytoplasmic form of 7ap can interact with cellular RNAs and may influence normal translational processes of the host cells for the benefit of the virus. The interaction of 7ap with intracellular proteins is also a possibility. It was reported that the similarly short and basic HIV-1 Tat protein exerts its regulatory effect through both nucleic acid and protein interactions.

Immunization of different species with Hu7ap failed to induce detectable antibodies against the protein. However, unsuccessful immunization is not unusual with small viral proteins and peptides. More interestingly, despite the species, the animals developed ANAs after immunization with Hu7ap. These experiments suggest an ability of Hu7ap to interact with or mimic autoantigenic macromolecules and to induce the breakdown of self-tolerance in different animals.

One possible explanation can be that 7ap itself is not immunogenic, but binds to DNA or nucleoprotein complexes released during and after injection from damaged tissue, and it may interfere with the normal DNA clearance processes mediated by serum amyloid P and C-reactive protein binding. Normally DNA and nucleoproteins are rapidly cleared from an injury site, and failure of clearance can lead to DNA or nucleoprotein specific B cell activation

6. New scientific results

1. We determined and analyzed the complete genome sequence and the phylogenetic localization of the PRRSV 9625/2012 isolate.
2. The sequence of PRRS-2 / Hungary / 102/2012 is the first European, wild, type 2 isolate, which is clearly not related to Ingelvac PRRS MLV
3. The transmembrane domains of GP2 and GP3 are encoded by the same nucleotide sequences as the signal peptides of GP3 and GP4 in the PRRSV. The transmembrane regions are conserved, but the overlapping signal peptides are always hypervariable.
4. The presence of a new ORF (ORF7a) was demonstrated in the genome of the virus with bioinformatic methods and the translation from ORF7a gene were verifying with the fusion protein constructs
5. ORF7a gene encodes a short, highly positive-charged peptide (7ap). 7ap peptides from PRRSV genotypes 1 and 2 have IgG, DNA and RNA binding ability, the 7ap binding site on IgG is located on the CH2 domain of the Fc portion.

6. The 7ap peptide from genotype 1 induces the production of nuclear antibody autoantibodies in the mouse and pigs.

7. Scientific Publications

Bálint Á, Balka G, Horváth P, Kecskeméti S, Dán Á, Farsang A, Szeredi L, Bányai K, Bartha D, Olasz F, Belák S, Zádori Z.: **Full-length genome sequence analysis of a Hungarian porcine reproductive and respiratory syndrome virus isolated from a pig with severe respiratory disease,** Arch. Virol., 160. 417-422, 2015. I.F: 2,058

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Olasz F, Dénes B, Bálint Á, Magyar T, Belák S, Zádori Z**.: Immunological and biochemical characterisation of 7ap, a short protein translated from an alternative**

frame of ORF7 of PRRSV, Acta Vet. Hung., 64. 273-287, 2016. I.F: 0,871

Olasz F, Dénes B, Bálint Á, Magyar T, Belák S, Zádori Z.: **Characterisation of the nucleic acid binding features of the PRRSV 7ap and its ability to induce antinuclear antibodies,** Acta Vet. Hung., 65. 124-134, 2017. I.F: 0,871

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Mészáros I, Tóth R, Olasz F, Tijssen P, Zádori Z.: **The SAT protein of porcine parvovirus accelerates viral spreading through irreversible ER stress induction,** J. Virol., doi:10.1128/JVI.00627-17, 2017. I.F: 4,606

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I.F: 0,212