

IMMUNOLOGICAL AND BIOCHEMICAL CHARACTERISATION OF 7ap, A SHORT PROTEIN TRANSLATED FROM AN ALTERNATIVE FRAME OF ORF7 OF PRRSV

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Sequence analysis revealed a short alternative open reading frame (ORF) named ORF7a within the nucleocapsid gene of genetically divergent porcine reproductive and respiratory syndrome virus (PRRSV) genomes. Alignment of the corresponding protein sequences (named 7ap) revealed substantial heterogeneity among 7aps of different genotypes, though all of them are predicted to be positively charged. Green fluorescent protein and FLAG fusion constructs of ORF7a of the HU-14432/2011 PRRSV demonstrated that 7ap is expressed. 7ap of HU-14432/2011 (Hu7ap) was synthesised chemically, and ELISA experiments revealed that Hu7ap binds strongly to mammalian IgGs. Protein-protein gel retardation assays and complement fixation inhibition suggest that 7aps bind to the CH2 domain of the IgG(Fc) fragment. Cellular localisation and immunological characteristics of PRRSV 7ap may indicate multiple functions including nuclear and cytoplasmic over-tuning of normal cellular processes and immunosuppression.

Key words: PRRSV, IgG binding, positive charged short peptide, overlapping ORF, complement fixation inhibition

Porcine reproductive and respiratory syndrome virus (PRRSV) is a member of the *Arteriviridae* family of the *Nidovirales* order. Since its appearance in the late 1980s PRRSV has remained one of the most costly diseases of the swine industry. PRRSV has a high mutation rate, which led to the evolution of two major genotypes with several subtypes (Murtaugh et al., 2010). The nucleotide identity between the two serotypes is only 55–70%. Type I PRRSV, also known as the European genotype, is prevalent in Europe (although it may also cause infections in Asia and North America), while type II, also known as the North American genotype, causes infections mainly in China and North America (Stadejek et

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al., 2006; Shi et al., 2010). The high genetic diversity of PRRSV hinders the development of protective measures against the virus (Mateu and Diaz, 2008). Despite extensive scientific efforts and the substantial financial investments by stakeholders, currently there is no effective vaccine against the virus on the market (Zuckermann et al., 2007; Charentantanakul, 2012; Tóth et al., 2016).

Several studies suggest that PRRSV can modulate the immune response of the host. Neutralising antibodies (NA) against PRRSV are generated in low levels in the early phases of the infection. Presence of non-neutralising antibodies in the early stages of the infection could enhance viral replication in alveolar macrophages due to antibody-dependent enhancement. The virus can evade the immune response of the host using several other strategies including delaying and inhibiting interferon response of the host cells, interfering with antigen presentation, masking neutralising epitopes by glycan shielding, and using decoy epitopes to prevent fast and effective humoral immune response (Lopez and Osorio, 2004; Mateu and Diaz, 2008).

The coding capacity of the PRRSV genome is maximised by an arrangement where the consecutive genes of the structural proteins are localised in alternating frames and they partially overlap with each other with the exception of ORF4 and ORF5 (Yu et al., 2009; Lunney et al., 2016). In addition, three accessory proteins were identified translating from fully overlapping ORFs by non-canonical translation mechanisms (Wu et al., 2001; Firth et al., 2011; Fang et al., 2012).

In this paper we report the identification by bioinformatic methods of a novel small protein (7ap) coding overlapping ORF (ORF7a) in the coding region of the N gene. Depending on the virus strain, the length of the protein is between 36 and 53 amino acids (aa). Translation of the ORF was proven by FLAG and GFP fusion constructs. 7ap binds DNA, RNA and the Fc part of the IgG, and it inhibits complement fixation *in vitro*. The potential functional implications of the findings are discussed.

Materials and methods

Fusion constructs

The pEGFP-N1 vector was used to fuse the three alternative frames of the nucleocapsid gene (ORF7) to GFP. In each construct approximately two thirds of the nucleocapsid gene of the PRRSV strain HU-14432/2011 was cloned, starting with the TRS sequence and ending with the last codon of the ORF7a. To amplify the cDNAs the 7F common forward primer was used. The three reverse primers 7r, 7ar and 7ncr differed only in the addition of single nucleotides to shift the translational frames in the three fusion constructs. The PCR products were digested with XhoI and BamHI and 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 (DYKDHDGDYKDHDIDYKDDDDK) into pcDNA3 by digesting the vector with XbaI and XhoI and inserting the annealed 3flagF and 3flagR oligonucleotides.

To generate similar fusion constructs in pcDNA-FLAG as it was done in pEGFPN1, the 7Ff forward and the 7Rf, 7arf and the 7ncrf reverse primers were used.

The mutation in the start codon of eGFP (M1A) was executed on the pEGFP-N1 vector with the gfp_mut_fw and the gfp_mut_rev primers using the quick change site directed mutagenesis method (Papworth et al., 1996). To express 7ap as a fusion protein in the resulted p-MeGFPN1 plasmid ORF7a was amplified with F-gfp-orf7a and R-gfp-orf7a primers, digested with XhoI and BamHI enzymes and cloned into the same sites of the plasmid (Table 1).

All constructs were transfected into Marc 145 (Kim et al., 1993) and PT (Bergeron et al., 1993) cells with Turbofect (Thermo Scientific, Waltham, Massachusetts) according to the manufacturer's recommendation on 24-well cluster plates.

Table 1

List of primers (the nucleotides causing frameshift are underlined)

Primers	Sequence
7F	GAGGATCCTTAACCTCGTCAAGTATGGCCG
7r	GACTCGAG <u>I</u> GGCGCACTGTATGAGCAA
7ar	GACTCGAG <u>TI</u> GGCGCACTGTATGAGCAA
7ncr	GACTCGAGGGCGCACTGTATGAGCAA
3flagF	TCGAGTGACTACAAAGACCATGACGGTGATTATAAAGATCAT GACATCGACTACAAGGATGACGATGACAAGTAGTGAA
3flagR	CTAGTTCACTACTTGTCATCGTCATCCTTGTAGTCGAT GTCATGATCTTTATAATCACCGTCATGGTCTTTGTAGCAC
7Ff	GACTCGAGTTAACCTCGTCAAGTATGGCCG
7Rf	GAGGATCC <u>I</u> GGCGCACTGTATGAGCAA
7arf	GAGGATCC <u>TI</u> GGCGCACTGTATGAGCAA
7ncrf	GAGGATCCGGCGCACTGTATGAGCAA
gfp_mut_fw	CCACCGGTCGCCACCGCGGTGAGCAAGGGCGAG
gfp_mut_rev	CTCGCCCTTGCTCACCGCGGTGGCGACCGGTGG
F-gfp-orf7a	CAACTCGAGATGACATCCGGCACCACCTC

Peptide synthesis

Hu7ap (MTSGTTSPLNAPSACNRSRRLSIKAQELRRFHPAGRSVFR LSLCCRLLIQCA) and Wu7ap (MTSGITLPLVSGNCVCRSRLPSIRVLELVP CQIQGG) were synthesised and purified to 95% purity by CASLO ApS (Lyngby, Denmark).

Indirect immunofluorescence (IF)

The cells were fixed with 300 μ l 3% formaldehyde solution and washed twice with 1 ml washing buffer [phosphate-buffered saline (PBS) 0.1% Tween 20]. The samples were then incubated in 200 μ l 1% Triton-X (in PBS) for 15 min at room temperature. Next the samples were washed with washing buffer and incubated with 200 μ l primary antibodies (mouse anti-FLAG M2 monoclonal antibody 1000-fold dilution (Sigma-Aldrich, St. Louis, Missouri) and serially diluted mouse and pig sera in IF buffer (4% horse serum in PBS). After incubation for 1 h, the cells were washed twice with washing buffer, and 200- μ l volumes of secondary antibodies [CF594 goat anti-mouse and CF568 goat anti-swine IgGs (Biotium Hayward, California) in 1000-fold dilution] and Hoechst 33342 (1 μ g/ μ l) were added. After washing, the samples were examined under a Zeiss Axio Observer D1 inverse fluorescence research microscope.

Direct ELISA

The plate was coated with 20 μ g/ml ORF7a solution and incubated overnight at 4 °C. Next day the plate was washed three times with washing buffer (0.5% Tween 20 in PBS), and blocked for an hour with 200 μ l blocking buffer (1% BSA/PBS solution) at 37 °C. The plates were then washed three times with washing buffer. The different HRP-conjugated antibodies [polyclonal swine anti-rabbit immunoglobulins/HRP (DAKO A/S, Glostrup, Denmark), goat anti-pig IgG (H/L) (AbD Serotec, Kidlington, UK), goat anti-chicken IgY(H+L)-HRP (SouthernBiotech, Birmingham, Alabama), rabbit anti-pig IgG H&L (HRP) (Abcam, Cambridge, UK), mouse anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Dallas, Texas)] were serially diluted with blocking buffer and added to the wells. After 1-h incubation at 37 °C the plates were washed four times and 100 μ l ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) solution (Ingenasa, Madrid, Spain) was added. After 20-min incubation the reaction was stopped with 5% SDS. OD was measured at 410 nm with an ELISA reader (ELx800 ELISA plate reader, Dialab GmbH, Austria). The samples were investigated in triplicates and the averages of the OD values were calculated.

Complement fixation inhibition test

To set up the complement fixation system, 25 μ l of 2.5% freshly prepared sheep erythrocytes (Culex Bt, Budapest, Hungary) was mixed with 25 μ l 1:2000 diluted haemolytic amboceptor (rabbit anti-sheep erythrocytes IgG) (Virion/Serion, Würzburg, Germany) and 25 μ l serially diluted (4–0.4% in ten steps) guinea pig complement (Virion/Serion, Würzburg, Germany), and the samples were incubated for 30 min at 37 °C. All dilutions of the components and the reactions were executed in veronal buffer (Idexx Laboratories, Westbrook, Maine). The lowest complement dilution where full lysis occurred was chosen and, to ex-

clude false positive results, one percent higher concentration complement was applied in the inhibition tests (so if full lysis was observed with 2% complement, then 3% was used in the inhibition test).

In the inhibition test staphylococcal protein A (spA) was used as negative control. All tested components including Hu7ap Wu7ap and spA were investigated in twofold serial dilution starting with 1.5 µg, 6 µg and 1.5 µg, respectively. For the test at first the haemolytic amboceptor was incubated for 1 h with the serially diluted peptides and spA at 37 °C in 25-µl volumes, then the other components were added in similar concentrations and volumes as indicated above. Samples were incubated again for 30 min at 37 °C and the results were analysed.

Protein-protein gel retardation assay

7ap was added to 8–10 µg IgG or IgG fragments in 14 µl 0.01 M potassium phosphate, 75 mM sodium chloride, pH 7.2 and was incubated for 1 h at room temperature, then 6 µl loading buffer was added and the samples were analysed by electrophoresis at 120 V for 2 h on a clear native polyacrylamide gel. The protein was stained with 0.1% Coomassie Brilliant Blue R250.

Results

Identification of a small conserved ORF

To identify unrecognised ORFs with potential translational products, bioinformatic examination of 46 sequentially divergent PRRSV genomes was performed.

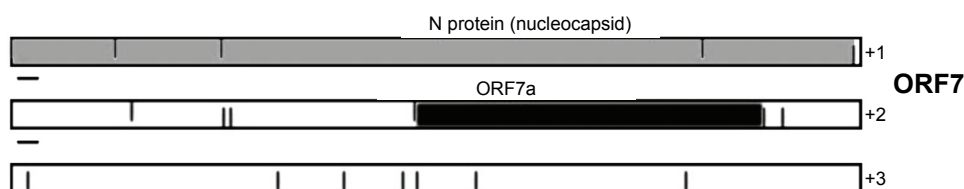


Fig. 1. Schematic representation of the three positive strand frames of the nucleocapsid coding region of PRRSV strain HU-14432/2011. The ORFs of interest are symbolised by shaded boxes.

+1 frame encodes the canonical protein (nucleocapsid), +2 frame encodes the ORF7a

Sequence analysis by the ORF finder tool (Tatusov) revealed a short alternative ORF named ORF7a (Fig. 1) within the nucleocapsid gene of all investigated virus genomes. ORF7a localised in the +2 frame containing a methionine codon in a conserved position and depending on genotypes continued in a coding region stretching between 26 and 53 aa. Alignment of the translated protein sequences (named 7ap) revealed five highly conserved and ten conserved aa in the

protein (Fig. 2). 7aps from divergent strains have different physicochemical properties; however, they are all predicted to be positively charged at neutral pH as a result of componential imbalance between basic and acidic aa in favour of the former one.

	* *	* **	* * ***	***	*** **	***	*** **	***
HKEU16 (EU076704.1)	MTSGTISPRP	SAPSACNRF	RLSIKAQELR	RFHPVGRSVF	RLSSCCRLLI	QCA		
KNU-07 (FJ349261.1)	MTSGITSPRL	NVPSACNRSR	RLSIKAQELR	RFHPVGRSVF	RLSSCCRLLI	QCA		
SD01-08 (DQ489311.1)	MTSGTISPKL	NAPSACNRSR	RLSIKAQELR	RFHPAGRSVS	RLSLCCRLLI	QCA		
BJEU06-1 (GU047344.1)	MTFGIISPTL	NAPSVCNRSR	RLSIKAQELR	RFHPVGRSVF	RLSLCCRLLI	QCA		
NMEU09-1 (GU047345.1)	MMSGITSPRP	SVLFACNRF	RLSIKVELR	RFHPVGRSVF	RSSCCRLII	QCA		
Amervac (GU067771.1)	MTFGTISPRP	NVPSACNRSR	RLLIKAQELR	RFHPAGRSVF	RLSSCCRLLI	QCA		
SHE (GQ461593.1)	MTFGTISPRP	NVPSACNRSR	RLLIKAQELR	RFHPAGRSVF	RLSSCCRLLI	QCA		
Lelystad (M96262.2)	MTSGTISPRL	NAPSACNRSR	RLSIKAQELR	RFHPAGRSVF	RLSLCCRLII	QCA		
HU-14432/2011	MTSGTISPRL	NAPSACNRSR	RLSIKAQELR	RFHPAGRSVF	RLSLCCRLII	QCA		
01CB1 (DQ864705.1)	MTSGTISPRL	NAPSACNRSR	RLSIKAQELR	RFHPAGRSVF	RLSLCCRLFI	QCA		
07v063 (GU737264.2)	MTFGTISPRL	SAPSACNRSR	RPSIKAQELH	RFHPVGRSVF	RLSSCCRLII	QCA		
IV3140 (DQ355822.1)	MMSGTISPRP	SAPFACNRSR	RLLIKAQELR	RFHPAGRSVF	RLSLCCRLII	QCA		
Lena (JF802085.1)	MMSGTISPEL	NVPSVCNRSR	QPLIRVQELR	RFHPAGRSVF	RLSLCCRSII	QCA		
Soz-8 (DQ324720.1)	MMSGTISHKL	NVLFACNRSR	PPLTRVQELR	RFRTAGRSVF	RLSSCCRSII	QCA		
Obu-1 (DQ324707.1)	MMSGTISPEL	NVFFVCNRSR	QPLIRVQELR	RFHPVGRSVF	RLSLCCRSII	QCA		
Yuz-48 (DQ324728.1)	MMSGTISPKL	NVLSACNRSR	RPSTRAQELR	RFHLAGRSVF	RLSLCCRSII	QCA		
Zap-41 (DQ324734.1)	MMSGTISPKL	NVLFVCNRSR	QPSTKAQELR	RFHPVGRSVS	RLSLCCRSII	QCA		
Vos-29 (DQ324725.1)	MMSGTISPKL	NVLFVCNRSR	QPSTKAQELR	RFHPAGRSVS	RLSLCCRSII	QCA		
Aus (AF438362.1)	MTSGITSPRL	NVPSVCNRSR	QPSIRVQELH	RFHPVGRSVF	R			
Pyrsvac-187 (DQ324712.1)	MTFGTISPRP	NVPSACNRSR	RLLIKAQELR	RFHPAGRSVF	RLSSCCRLII	QCA		
Upa-13 (DQ324721.1)	MTSGTISPRL	NAPFACNRSR	RLSIKA					
Dzi-64 (DQ324706.1)	MTFGTISPRL	SVFFACNRSR	RPLIKAQELH	RFHPVGRSVF	RLSLCCRLII	QCA		
Che-46 (DQ324703.1)	MTFGTISPKP	NVFFACNRSR	QPLIKVQELR	RFHPVGRSVF	RWSSCCRLII	QCA		
Vas-3 (DQ324723.1)	MTFGTISPKL	SVFFVCNRSR	QPSIRAQELR	RFHPVGRSVF	R			
Zad-39 (DQ324733.1)	MMSGTISPEL	NVFFVCNRSR	QPLIRVQELR	RFHPAGRSVF	RLSLCCRSII	QCA		
Sno-6 (DQ324714.1)	MTSGIISPQL	SVLFVCNRSR	QPLIRVQGLH	RFHLAGRSVF	RWSSCCRLII	QCA		
WUH4 (JQ326271.1)	MTSGITLPLV	SGNCVCRRSR	LPSIRVLELV	PCQIQGG				
VR-2332 (AY150564.1)	MMSDITLPLV	SGNCVCRQSR	PPLIKALGLA	PCQIQGG				
MN30100 (EF536000.1)	MTSDITLPLV	SGNCVCRRSR	LLLIKALELA	PCQIQGG				
NVDC-JS2-2011 (JQ715698.1)	MTSGITLPLV	SGNCVCRRSR	LPSIRALELV	PCQIQGG				
NVSL 97-7985 (AF325691.1)	MTSDITLPLV	SGNCVCRQSR	LPLIKALELA	PCRIQGG				
CH-1a (AY032626.1)	MTSGITSPLV	SGNCVCRRSR	LPLTRAPELV	PCQIQGG				
Hawkeye 7 (EF532815.1)	MTLDITSPLV	SGNCVCRQSR	LPLTKALELV	PCRIQGG				
Flagship (EF532805.1)	MTLDITSPLV	SGNCVCRQSR	LPLTKALELV	PCRIQGG				
JA-142 (AF396844.1)	MTSGITSPLV	SGNCVCRRSR	LPLTRALEPV	PYQIQGG				
Yn-2011 (JX857698.1)	MMSDITLPLV	SGNCVCRQSR	PPLIKALGLA	PCQIQGG				
A2MC2 (JQ087873.1)	MMSDITLPLV	SGNCVCRQSR	PPLIKALGLA	PCQIQGG				
MN184B (DQ176020.1)	MTLDITSPLV	SGNCVCRQSR	LPLTKALELV	PCRIQGG				
SDSU73 (JN654458.1)	MTSDITLPLV	SGNCVCRQSR	PPLIKALELA	PCRIQGG				
P129 (AF494042.1)	MTSGITSPLV	SGNCVCRRSR	LPLTRALELV	PCQIQGG				
17198-6 (EF442776.1)	MTLDITSPLV	SGNCVCRQSR	LLLIKALELA	PCQIQGG				
CA (FJ194950.1)	MMSDITLPLV	SGNCVCRQSR	PPLIKALGLA	PCQIQGG				
05K212 (EF441810.1)	MTSDITLPLV	SGNYVCRQSR	LPLTKALELA	PCQIQGG				
PL97-1/LP1 (AY612613.1)	MMSDITLPLV	SGNCVCRQSR	PPLIKALGLA	PCQIQGG				
JXwn06-81c (HQ233604.1)	MTSGITLPLV	SGNCVCRRSR	LPSIRALELV	PCQIQGG				
SX-1 (GQ857656.1)	MTSGITLPLV	SGNCVCRRSR	LPSIRALELV	PCQIQGG				
	** **	*****	* **	*** **				

Fig. 2. Alignment of 7ap sequences. Accession numbers of the different strains are in parentheses. Amino acids in bold are conserved in both genotypes, the asterisks in the first and last rows label conserved amino acids in type I and type II sequences, respectively

Translation and localisation of 7ap

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. To demonstrate the existence of 7ap and to investigate the translation mechanism of ORF7a eGFP fusion constructs were created by cloning the cDNA of the 5' half the ORF7 mRNA (starting

with the translational regulator sequence and finishing with the last codon of 7ap) of the HU-14432/2011 PRRSV strain into the pEGFPN1 vector in the three consecutive frames (Fig. 3A–B). The constructs were transfected into Marc-145 cells and, after 24 h, the cells were fixed and monitored for eGFP expression. As expected, the positive control construct, in which the eGFP was inserted into frame +1 coding the nucleocapsid protein, resulted in a robust fluorescent signal in the cells. Also, strong fluorescent signal was detected, although with lower intensity, in the PT cells when eGFP was fused to the last codon of 7ap in frame +2 indicating the translation of ORF7a. Interestingly, low levels of eGFP signal could also be detected at high-intensity excitation in cells transfected by the fusion constructs containing the eGFP in frame +3 (Fig. 3C).

To confirm the translation of 7ap and to exclude that the detected fluorescent signal in the frame +2 construct was the result of artificial initiation of the eGFP translation from its own ATG codon, other plasmid constructs were generated, where the eGFP fusion tag in the three different frames was replaced by a triple FLAG tag. These constructs were also transfected into PT cells, which were monitored for FLAG expression 28 h post transfection. FLAG expression could be detected in cells transfected by the frame +1 and frame +2 constructs, verifying that 7ap is in fact translated from the nucleocapsid mRNA (Fig. 3D).

To facilitate background-free tracing of 7ap in the cell, first the pEGFPN1 vector (p-MeGFPN1) was modified by mutating the start codon of eGFP from ATG (M) to GCG (A). This mutation prevented the translation of eGFP from its own start codon (M1AeGFP), but allowed initiation from a fusion partner and the translation of M1AeGFP as a fluorescently active fusion protein. Second, ORF7a was cloned into p-MeGFPN1 by fusing 7ap to M1AeGFP (7ap-M1AeGFP). This plasmid construct was transfected into Marc 145 cells and the 7ap-M1AeGFP signal was followed in the transfected cells. Soon (16–20 h) after transfection, the majority of the fluorescent signal appeared in the nucleus, while later it could be detected in the whole cell, indicating that 7ap is first transported into the nucleus and later it also accumulates in the cytoplasm (Fig. 3E).

IgG binding and inhibition of complement fixation

For serological detection of 7ap ORF7a of the HU-14432/2011 strain was expressed in *E. coli* by two different expression systems, pet28b (Merck KgaA, Darmstadt, Germany) and pBAD2TEV (Zádori et al., 2001). In both systems the expressed protein proved to be highly toxic to the bacteria and purification of bacterially expressed 7ap remained unsuccessful despite several attempts (data not shown).

To complete the serological investigation of PRRSV-positive animals, 7ap of HU-14432/2011 (Hu7ap) was synthesised chemically and used for coating the plate in a standard indirect ELISA test. PRRSV-positive sera (they were found positive in 1000- to 2000-fold dilution by INGEZIM PRRS Universal kit) were

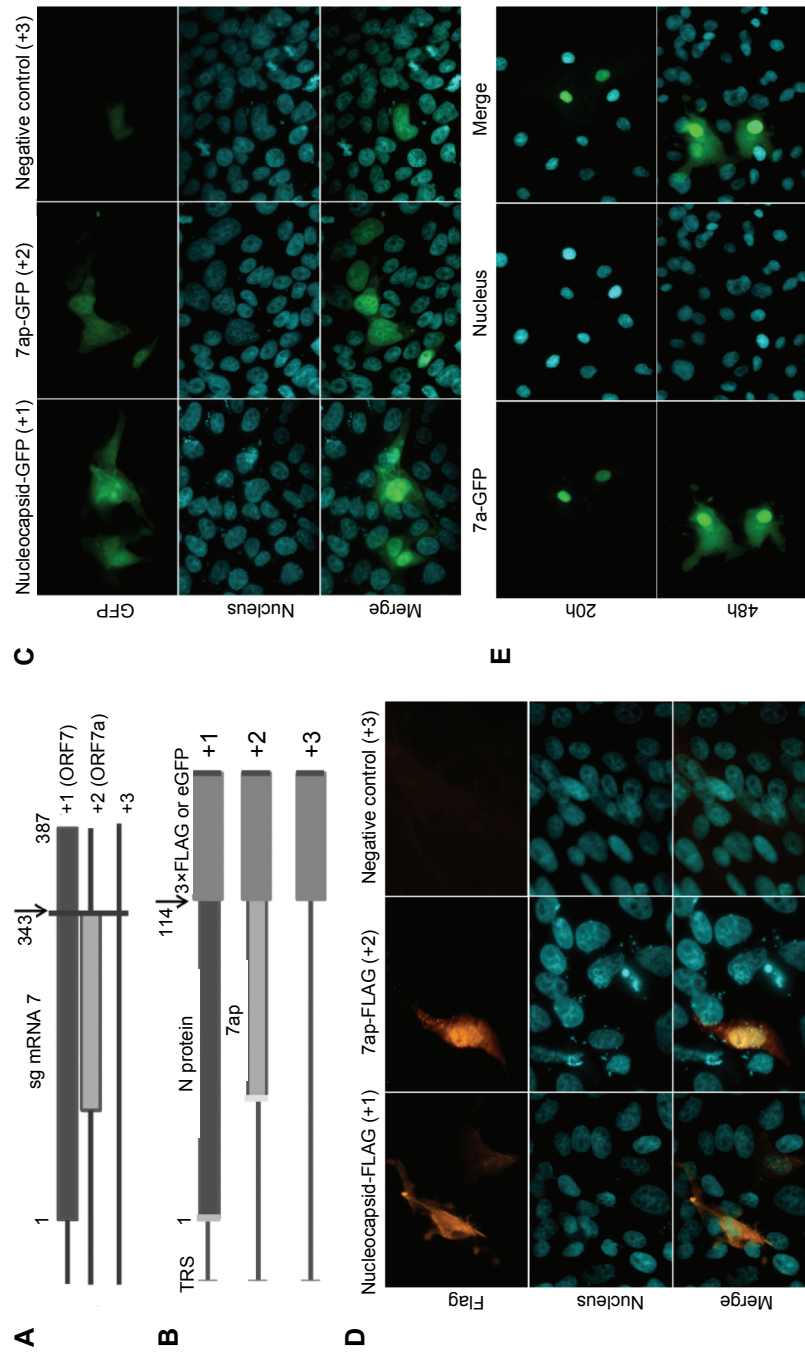


Fig. 3. Translation and topology of the 7ap fusion proteins. (A) Schematic representation of the three frames of mRNA 7. In the fusion constructs ORF7 was truncated at nucleotide position 343 of the N gene at the end of ORF7a. (B) 3xFLAG and eGFP protein was fused to amino acid 114 of the N protein and to the last amino acid of 7ap while the +3 frame did not contain ORF. (C) Translation of the eGFP fusion constructs and localisation of the proteins in PT cells. (D) Translation of the 3xFLAG fusion constructs and localisation of the proteins in PT cells. (E) Changes in the localisation of 7ap-eGFP with time in Marc 145 cells. Elapsed times after transfection are indicated

produced by intranasal infection of four pregnant sows with the HU-14432/2011 (GenBank accession number: KR296711.1) pathogenic strain. In the experiment, the sera of a non-infected pig and Hu7ap-coated wells without primary antibody treatment were also probed as standard negative controls.

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 [F(ab)], independent of the IgG binding capability of 7ap.

To explore the nature of binding, several other mammalian IgG HRP conjugates were also investigated by ELISA. These 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 (Table 2).

Table 2

IgG binding capability of 7ap measured by direct ELISA

IgG-HRP conjugates	Dilutions of sera				
	1000×	2000×	4000×	8000×	1000× not 7ap coated
Goat anti-chicken IgG	++	+	+/-	-	-
Goat anti-pig IgG	+++	++	+	+	-
Swine anti-rabbit IgG	+	+	+/-	-	-
Rabbit anti-pig IgG	++	+	+/-	-	-
Mouse anti-rabbit IgG	++	+	+	+/-	-
HRP	-	-	-	-	-

+++ : OD > 1.6, ++ : OD = 1.59–0.8, + : OD = 0.79–0.4, +/- : OD = 0.39–0.2, - : OD < 0.19

Protein-protein gel retardation assay (Park and Raines, 2004) was performed with Hu7ap to confirm the results of ELISA and to localise the binding site/s of 7ap on swine IgG. To explore whether a sequentially divergent 7ap possesses similar binding characteristics as Hu7ap, the 7ap of a type II PRRSV strain, WuH4 (Wu7ap) was also synthesised and analysed. Both Hu7ap and Wu7ap are positively charged peptides (pI 11.7 and 9.3, respectively) at near neutral pH, so under native electrophoretic conditions they move in the opposite direction (toward the cathode) than the negatively charged IgGs and their fragments. The interaction between the negatively charged proteins and the positively charged peptides may result in complete inhibition of gel entry of the negatively charged protein as a consequence of increased size and decreased net negative charge of the complex.

In fact, 4 µg and 20 µg of Hu7ap and Wu7ap were able to completely inhibit the entry of 10 µg of the monoclonal mouse IgG into the gel and twice as much was needed (8 µg and 40 µg, respectively) to titrate out 10 µg polyclonal pig IgG.

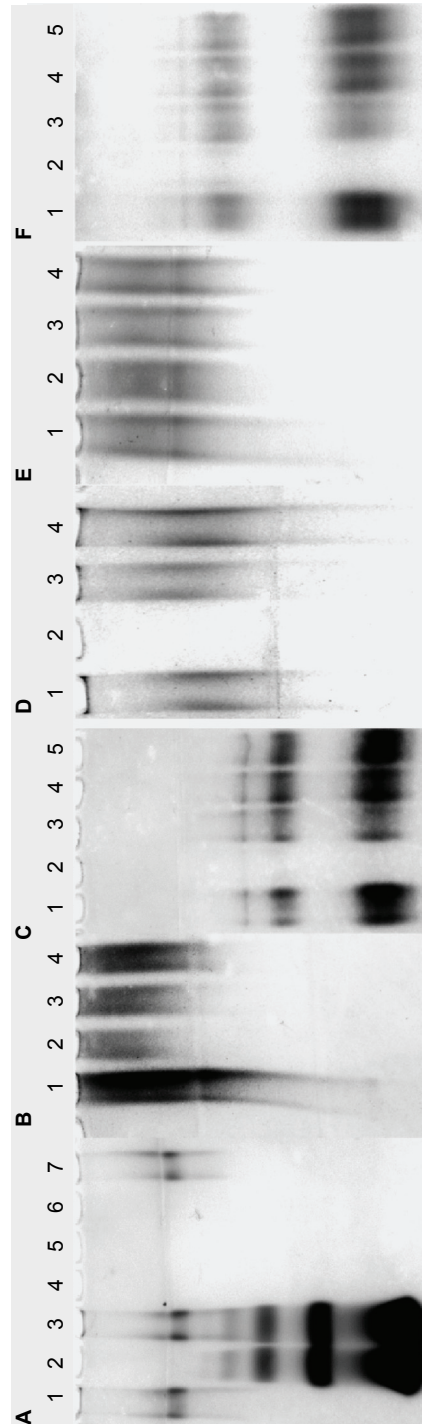


Fig. 4. Antibody-7ap gel retardation assays. (A) Monoclonal mouse IgG-Hu7ap interaction. Lanes 1-3 serve as negative controls. Lane 1: 10 µg IgG; Lane 2: 80 µg BSA; Lane 3: 10 µg IgG+80 µg BSA; Lanes 4-7: 10 µg mouse IgG + Hu7ap in bisecting dilution (16 µg, 8 µg, 4 µg and 2 µg, respectively). (B) Polyclonal pig IgG (Fab)₂-Hu7ap interaction. Lane 1: 8 µg IgG (Fab)₂; Lanes 2-4, 8 µg IgG(Fab)₂ + Hu7ap in bisecting dilution (32 µg, 16 µg, 8 µg, respectively). (C) Polyclonal pig IgG(Fc) fragment-Hu7ap interaction. Lane 1: 10 µg IgG(Fc); Lanes 2-5: 10 µg IgG(Fc) + Hu7ap in bisecting dilution (16 µg, 8 µg, 4 µg, 2 µg, respectively). (D) Polyclonal pig IgG-Wu7ap interaction. Lane 1: 10 µg IgG; Lanes 2-4: 10 µg IgG + Wu7ap in bisecting dilution (20 µg, 10 µg, 5 µg, respectively). (E) Polyclonal pig IgG(Fab)₂ - Wu7ap interaction. Lane 1: 10 µg IgG (Fab)₂; Lanes 2-4, 10 µg IgG (Fab)₂ + Wu7ap in bisecting dilution (50 µg, 25 µg, 12.5 µg, respectively). (F) Polyclonal pig IgG(Fc) fragment-Wu7ap interaction. Lane 1: 8 µg IgG(Fc); Lanes 2-5: 8 µg IgG(Fc) + Hu7ap in bisecting dilution (32 µg, 16 µg, 8 µg, 4 µg, respectively).

Similarly, applying in increasing concentrations 16 µg and 32 µg of Hu7ap and Wu7ap titrated out completely 10 µg 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 (Fig. 4). 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 localised on the Fc fragment.

To further characterise the 7ap binding site, additional experiments were performed. SpA binds to the CH3 domain of the Fc fragment while C1q of the complement system binds to the CH2 domain. In a competitive ELISA test 7aps applied in high concentrations did not inhibit spA-HRP binding to polyclonal pig Ig(Fc) (data not shown). However, in a standard complement fixation experiment, when anti-sRBC rabbit IgG (haemolysin) was preincubated with Hu7ap and Wu7ap, concentration-dependent inhibition of the haemolysis by the former one was observed, indicating the interference of Hu7ap with C1q binding to Fc (Table 3). These experiments suggest that Hu7ap binds to the CH2 domain of the Fc, and its binding site at least partially overlaps with the C1q binding site.

Table 3

Complement fixation inhibition of different proteins

Proteins	Concentration, ng/µl	Dilutions of proteins			
		1×	2×	4×	8×
Hu7ap	15	+	+	+	–
Wu7ap	60	–	–	–	–
spA	15	–	–	–	–

+: inhibition, –: no inhibition

Discussion

The nucleotide identity between the two PRRSV serotypes is only 55–70% and, according to one calculation, the mutation rate of PRRSV is $4.7\text{--}9.8 \times 10^{-2}$ /nucleotide/year (Jenkins et al., 2002; Hanada et al., 2005), which would make it the highest reported among RNA viruses. However, more conservative estimations put the mutation rate somewhere between 1.8 and 7×10^{-3} /nucleotide/year (Chang et al., 2002; Forsberg, 2005), which still places PRRSV among the most rapidly evolving viruses known. Despite the rapidly changing codon sequences of the main ORFs, recently several positionally conserved alternative ORFs have been identified in the PRRSV genome and all of them proved to be protein coding: ORFs partially coding the nsp2TF and nsp2N proteins in the NSP2 region (Fang et al., 2012), ORF5a and ORF2b overlapping with the ORF5 and ORF2a

genes (Wu et al., 2001; Firth et al., 2011), respectively. Genome comparison of 46 divergent PRRSV genomes revealed another short ORF in a conserved position, named ORF7a overlapping with the ORF7 gene. Study of GFP and FLAG fusion constructs of the cDNA of the ORF7 region of the HU-14432/2011 PRRSV strain 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 (Meier et al., 2006), bovine coronavirus 1 (Senanayake and Brian, 1997) and mouse hepatitis virus (MHV) (Fischer et al., 1997)] belonging to the genus *Betacoronavirus* was reported previously. The so-called I protein of MHV is a structural protein and its absence leads to reduced viral growth in tissue culture (Fischer et al., 1997), while ORF-9B of SARSV localises to mitochondria, binds to PCBP2 and AIP4, induces autophagy and severely limits host cell IFN responses (Shi et al., 2014). Overprinting of the nucleocapsid gene with an alternative ORF is not restricted to nidoviruses, since it can also be found at the core protein of the hepatitis C virus [Alternate Reading Frame Protein (ARFP)]. HCV ARFP stimulates apoptosis in plasmacytoid dendritic cells (Samrat et al., 2014), and it induces a cytokine imbalance in favour of immunosuppression by inhibiting IFN- α secretion and the production of IL-10 (Xu et al., 2014). Although the function of the alternative frame proteins is different even in related viruses, they all appear to promote viral spreading either directly as viral proteins or indirectly as immunosuppressive accessory proteins.

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. On the C1q heterotrimer several basic residues (7 arginines and one lysine and histidine each) have been identified to participate in residue-residue interaction with the Fc domain of IgG (Schneider and Zacharias, 2012). In addition, Arg^{B114} and Arg^{B129} of the C1q complex were proposed to mediate IgG recognition (Marqués et al., 1993). 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 neutralisation by interaction with the components of the complement pathway (Hulo et al., 2011a). 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) (Hulo et al., 2011b). However, to our knowledge PRRSV 7ap is the first reported viral protein which binds to the Fc domain of IgG and inhib-

its complement activation. Presently it is not clear when and where 7ap interacts with IgG during viral infection. We were unable to detect *in vitro* secretion of the 7ap-eGFP fusion protein from the supernatant of transfected cells by fluorimetric measurements (data not shown). However, externalisation by cell lysis or *in vivo* secretion of 7ap from infected macrophages cannot be excluded and it may ensure interaction.

Direct interactions of 7ap with intracellular proteins cannot be excluded either, particularly because most of the cytosolic proteins have net negative charges (Schwartz et al., 2001), while several regulating proteins interacting with them, like 7ap, are significantly shorter and have strong basic characters (Kiraga et al., 2007). It was shown that HIV-1 Tat, a similarly short viral basic protein, is able to bind to several regulator proteins and its binding to the cytosolic I κ B- α depends on the arginine-rich region of the protein (residues 48–60) (Vitagliano et al., 2011).

During its evolution, PRRSV developed several molecular tools to counteract host responses and to evade the host immune system. Localisation and the remarkable immunological features of 7ap indicate that this protein is also one of such viral tools. Although our research revealed a substantial amount of data about the biochemical characteristics of 7ap, further *in vitro* and *in vivo* studies are needed to clarify its translation in macrophages and its functional role in the life cycle of the virus.

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