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1 **Genetic diversity of porcine reproductive and respiratory syndrome virus**
2 **strains circulating in Hungarian swine herds**

3
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20 Keywords: PRRSV, phylogenetic analysis, glycosilation

24 Note: Nucleotide sequence data reported are available in the GenBank databases under the
25 accession numbers: DQ3666339 – DQ3666358, and EF406336 – EF406352.

26 **Abstract**

27

28 Analysis of 37 ORF5 sequences of Hungarian porcine respiratory and reproductive syndrome
29 virus (PRRSV) strains revealed that most of them (35) belonged to the European genotype,
30 forming distinct subgroups, reflecting the exceptional diversity of Eastern European strains.
31 Twelve vaccine-like strains were also found in non-vaccinated animals. Two strains belonged
32 to the American genotype showing 90-91% nucleotide identity to the “Quebec” Canadian
33 reference strain. The analysis of the putative ectodomains and their N-linked glycosylation
34 sites of the vaccine strain and its variants suggested selective pressure on the first ectodomain,
35 by a consistent amino acid change on epitope B and by losing a glycosylation site in the
36 otherwise conserved N-46 position.

37 1. Introduction

38 Porcine respiratory and reproductive syndrome (PRRS) is a widespread disease of
39 swine characterised by reproductive disorders in gilts and sows and by respiratory signs,
40 leading to death mostly in neonatal, suckling and weaned piglets. The porcine respiratory and
41 reproductive syndrome virus (PRRSV) is an enveloped, single stranded RNA virus of the
42 *Arteriviridae* family, member the order *Nidovirales* (Cavanagh et al., 1997). The genome of
43 PRRSV is approximately 15 kilobases (kb) in length, and comprises nine open reading frames
44 (ORFs). ORF 1a and 1b are coding for the enzymes responsible for the replication, ORF2a,
45 and ORFs 3 to 5 are coding the membrane associated glycoproteins, ORF2b and ORF6 are
46 encoding the nonglycosylated membrane proteins and the ORF7 codes for the nucleocapsid
47 protein (Wu et al., 2001; Snijder and Meulenberg 1998).

48 One of the most variable region of the PRRSV genome is the ORF5 encoding the 25-
49 kDa glycoprotein 5 (GP5) (Andreyev et al., 1997). This glycoprotein forms a heterodimeric
50 complex with the M protein (encoded by ORF6) via disulphide bound and is located in the
51 membrane of the virion (Snijder et al., 2003). GP5 contains neutralization epitopes in its N-
52 terminal ectodomain and is one of the targets of the protective anti-viral immunity, since
53 antibodies produced against it can protect the animals from viraemia and the development of
54 the characteristic PRRSV lesions (Pirzadeh and Dea 1997; Balasuriya and MacLahan 2004).

55 Various sequence analyses have proven that there are marked genetic differences
56 between the two major genotypes, the European and North American strains (Meng et
57 al., 1995). In the past, European isolates were considered to be less variable than the American
58 strains, however, recently significant differences were detected among the Eastern European
59 strains supporting the definition of new genetic subtypes (Forsberg et al., 2002, Pesch et al.,
60 2005, Stadejek et al., 2006). Genotypes forming a unique cluster were found in Lithuania and

61 thought to be closely related to the common ancestors of the European and American strains
62 (Stadejek et al., 2002)

63 In Hungary only the presence of the European type sequences were reported so far
64 (Medveczky et al., 2001; Kiss et al., 2006). Considering the high diversity of the Eastern
65 European strains (Stadejek et al., 2002, 2006), including those surrounding Hungary (Indik et
66 al., 2005), and the observation that genetic variability might affect vaccination efficacy
67 (Labarque et al., 2004); the aim of this study was to detect and characterise PRRSV strains in
68 Hungary, and by comparing their ORF5 sequences, analyse their relationship to other
69 European and American isolates. Since European-type live virus vaccines are widely used in
70 Hungary, the presence of vaccine virus-like sequences in non vaccinated pigs of vaccinated
71 herds and their genetic stability was analysed by comparing them to the original vaccine
72 strains.

73

74 **2. Materials and methods**

75 *2.1. Sample collection*

76 Samples (lungs, were collected from pig farms located in different parts of Hungary,
77 between 2003 and 2006. (Description of the samples analysed in this study is given in Table
78 1.)

79

80 *2.2. RNA extraction and RT-PCR*

81 The RNA preparation from the supernatant of the centrifuged tissue homogenates,
82 and from the sera and semen samples was carried out using the QIAmp Viral RNA Mini Kit
83 (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA was
84 stored at -80°C until used.

85 The “diagnostic” primer pair (forward: 5’-CAGCCAGTCAATCARCTGTG-3’ and
86 reverse 5’-TCGCCCTAATTGAATAGGTG-3’) was designed to amplify a part of the ORF7
87 and the 3’ noncoding region (from nucleotide 14658 to 15050 on the Lelystad strain,
88 accession number: M96262, and 14937 - 15364 on the American reference strain VR-2332,
89 acc. number: NC_001961) and to be used for detecting both European and, if present,
90 American sequences in clinical samples. The positive samples were further tested by
91 amplifying the ORF5 sequences using primers 5’-GTTGCTSCATTTTCMTGACAC-3’ and 5’-
92 ATCGTCTAGGCCTCCCATG-3’ for the European strains (13416 – 14104 on the Lelystad
93 strain); and 5’-ACCATGAGGTGGGCAACTGT-3’ and 5’-
94 TGGAGCCGTGCTATCATGAC-3’ for the American strains (13721 – 14419 on VR-2332).

95 The reverse transcription reactions (RTs) and the amplifications were performed by a
96 continuous RT-PCR method using the Qiagen One-Step RT-PCR Kit (Qiagen, Hilden,
97 Germany) at 52° annealing temperature for 35 cycles.

98

99 *2.3. Sequencing and analysis of the sequence data*

100 The amplicons were sequenced using an ABI PRISM 3100 automatic sequencer. The
101 chromatograms were visualised with the Chromas 2. software, and they were identified with
102 BLAST (NCBI, Bethesda, USA), aligned by ALIGN PLUS 4 for Windows 95, version 4.0.
103 Phylogenetic analyses were performed using the CLUSTAL X 1.81 software employing IUB
104 DNA weight matrix with 0.5 transition ratio. Bootstrap resampling was carried out on 1000
105 replicate data sets. Phylogenetic trees were plotted with the TREEVIEW (Win32 version
106 1.6.6.) software. The N-linked glycosylation sites were determined with the NetNGlyc 1.0
107 server software (www.expasy.ch).

108

109

110 3. Results and discussion

111 Large majority of the samples, that were positive after the “diagnostic”, ORF7 RT-
112 PCR were sequenced after amplifying a part of the ORF5 gene with the appropriate primers.
113 Altogether 37 ORF5 sequences were analysed obtained from 17 herds all over the country.
114 The nucleic acid sequences were aligned and compared to each other and to selected
115 European and American strains. 35 of the 37 Hungarian strains belonged to the European
116 genotype showing 92.54±1.93% average nucleotide identity (and standard deviation) to the
117 Lelystad reference strain in the corresponding region of the genome. Strains HU12 and HU21
118 showed 86.80%, and 85.87% nucleotide identity with the American reference strain VR-2332,
119 respectively.

120 In one case (HU13), porcine alveolar macrophage (PAM) culture was prepared from
121 the lungs of an apparently healthy piglet for further virus propagation. To test the cell culture
122 before inoculation RNA was prepared from it and gave positive RT-PCR reaction when
123 applying the “diagnostic” primers, hence confirmed the “genuine” PRRSV infection of the
124 PAM cells. The aligned ORF5 sequence of the isolate from the PAM cells of the piglet
125 differed only by one nucleotide, and one AA (g₁₃₆₅₉ to a, resulting D₅₆ to U) from a European-
126 type live vaccine strain. The mother of the piglet was vaccinated twice during gestation with
127 this vaccine. Two other strains (HU14 and HU19) recovered from the same herd from
128 healthy, non-vaccinated gilts, housed together with vaccinated sows had 98.84% nucleotide
129 identity, 97.92% and 98.61% amino acid identity with the vaccine virus, respectively.

130 In an other case, amplicons from different positive samples collected in a seropositive
131 herd were sequenced. In the herd two different European-type live vaccines were used one
132 after the other (first “vaccine a”, then “vaccine b”, then “vaccine a” again). The samples were
133 obtained in the third phase when “vaccine a” was used again. Analysing the results of the
134 nucleotide sequencing we could identify strains showing 98.84-99.77% nucleotide identity

135 with “vaccine b”. All of these samples were lungs of aborted fetuses or piglets showing
136 severe respiratory symptoms before death. None of the piglets were vaccinated with any type
137 of vaccine, only their mothers were vaccinated twice with “vaccine a” during their gestation.
138 It is remarkable that “vaccine b” was detected from all the pigs 3-4 months after re-
139 introduction of “vaccine a” (Kiss et al., 2006).

140 A phylogenetic tree was constructed by using sequence data of amplicons from 71
141 strains: 37 Hungarian sequences, and a further 34 representatives of different genogroups,
142 deposited in the GenBank (Fig. 1). Strains used for the construction of the phylogenetic tree
143 are shown in Table 1.

144 In the phylogenetic tree the separation of the Hungarian strains resembling European
145 and American type PRRSV genotypes was supported by high bootstrap values: 35 of 37
146 strains were positioned among the European genotype, while strain HU12 and HU21 were
147 clustered within the American genotype. The Hungarian strains of the European genotype are
148 all clustered within Subtype 1 defined by Stadejek et al., (2006), and located in four different
149 subgroups, while one strain (HU16) was positioned alone. Subroup 4 was formed by 20
150 Hungarian strains, 54% of the strains investigated in this study. Within this subgroup the
151 separation of the strains was in correlation with the distance of the geographical location of
152 the herds where these samples were collected. All vaccine related strains of subgroup 1 and 2
153 were recovered from herds using European type live-virus vaccine. None of these animals
154 were vaccinated with any type of vaccine. These results suggested that the live vaccine-virus
155 strains were able to spread and circulate within the herd. Strain HU12 and HU21 are the first
156 identified strains in Hungary belonging to the American genotype. In Hungary the use of
157 American type vaccine is not authorised, and HU12 was detected in a herd which has
158 breeding contact with a Danish farm (boars are regularly introduced from Denmark).
159 Although in Denmark both genotypes are reported to be present (Madsen et al., 1998), the

160 origin of these strains is unknown because they are more similar to the “Quebec” Canadian
161 reference strain (90%, and 91% nucleotide identity) than any American type MLV strain. The
162 herd where HU21 was detected is fattening weaned piglets of herd where HU12 was found.

163 Analysing the nucleic acid and the deduced amino acid identity values of the vaccine
164 related strains compared to live vaccine virus strains it is remarkable that in case of the
165 derivatives of “vaccine b” all amino acid changes were found in the putative ectodomain,
166 consistently at the same amino acid positions (Fig. 2). The AA change at position 37 may also
167 affect the attachment of neutralizing antibodies, since this is the first amino acid of the
168 neutralizing epitope B (Balasuriya and MacLahan 2004; Ostrowski et al., 2002). Analysing
169 the putative N-linked glycosylation sites of the first ectodomain of the live vaccine virus
170 strains and their derivatives, it was found that in case of “vaccine b” almost all the vaccine-like
171 variants lost the N-46 glycosylation site, by a consistent N to K AA change (compared to the
172 vaccine strain). Variability in this region is rare; since this part of the ectodomain is thought to
173 be extremely conserved (Pesch et al., 2005), and only few reports are found on wild type
174 strains without N-46 (Mateu et al., 2006; Stadejek et al., 2006). Vaccine-like strains lacking
175 N-46 are not reported so far. Strain HU08 is also without N-46. In an *in vitro* study the
176 infectivity of mutant PRRS viruses lacking oligosaccharide bound to N-46 exhibited a
177 significantly reduced infectivity compared to the wild type virus due to improper folding of
178 the GP5, which led to inefficient GP5-M heterodimerisation (Wissink et al., 2004). Loss of
179 glycan residues of the GP5 ectodomain enhances both the sensitivity of the viruses to *in vitro*
180 neutralization and the immunogenicity of the nearby neutralization epitopes (Ansari et al.,
181 2006). However all these strains lacking N-46 have a glycosylation site at AA position 37 (the
182 vaccine has N-35), which is thought to adopt the function of N-46 (Wissink et al., 2004). The
183 comparison of these variants to the parental, wild isolate, from which the vaccine was
184 prepared could possibly confirm the reversion of the vaccine, because all “vaccine b”-like

185 sequences were recovered from aborted fetuses, or carcasses having severe respiratory
186 problems prior to death (Table 1), and the economical losses caused by PRRS-like symptoms
187 have extremely elevated after the introduction of this vaccine. Furthermore in an other herd
188 where “vaccine a” was used alone the AA changes of the vaccine like strains were found in
189 random distribution, all the “vaccine a”-like sequences were obtained from clinically healthy
190 animals, and such losses were not observed within the herd. Comparing the nucleotide and the
191 amino acid sequence alignments it is also remarkable that the incidence of the synonymous
192 mutations in case of the vaccine-like strains is quite low. One might speculate that the
193 explanation of this phenomenon could be the selective pressure of the immune system
194 directed against the GP5 ectodomain, and indicates putative positive and negative selection
195 sites on this part of the genome as observed previously by other investigators (Storgaard et al.,
196 1999; Mateu et al., 2006). To avoid this selective pressure, those variants that have developed
197 greater phenotypic differences (AA changes) compared to the original strain, had better
198 chance to spread and persist within the herd while silent mutations were not rewarded by
199 higher chances to multiply and spread. Detailed sequence analyses are needed in the future to
200 identify the positive and negative selection sites, to verify the possible reversion of the
201 vaccine virus or determine the cause of the increased losses after the introduction of the
202 vaccine.

203

204 **4. Acknowledgements**

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1 **Figure legends**

2 Table 1. Viruses involved in the genetic investigations.

3

4 * Porcine alveolar macrophage culture prepared from an apparently healthy 10 day old piglet for purposes
5 of virus propagation but tested positive by PCR before inoculation

6

7

8 Figure 1. Phylogenetic tree based on the data of nucleotide sequences on the ORF 5 gene of 37 Hungarian
9 strains and 34 foreign strains. Abbreviations are indicated in Table 1. Bar on the right demonstrates the
10 genetic distance. Internal labels represent the bootstrap values of 1000 replicates.

11

12

13 Figure 2. Multiple alignment of the putative amino acid sequences of the vaccine derived PRRSV strains
14 between amino acid position 33 and 176 of the GP5 protein.

15

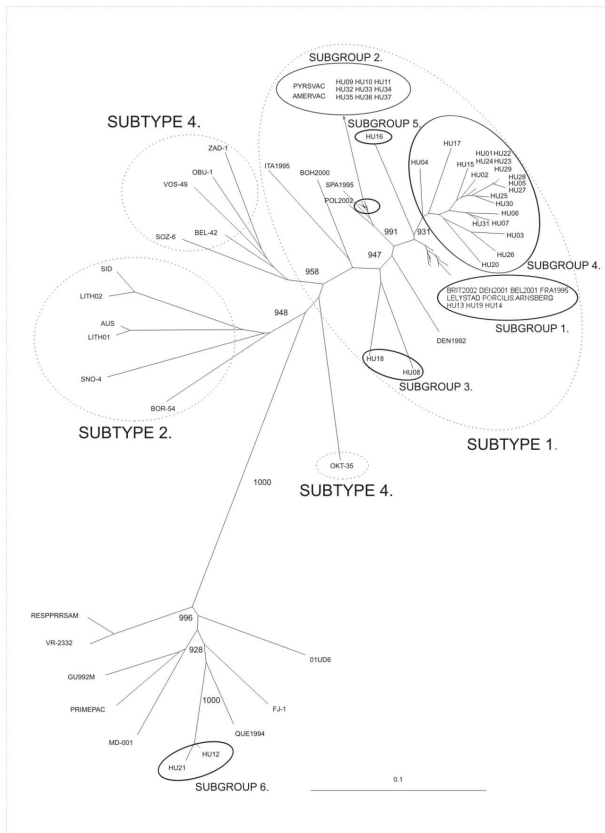
16 Bars under the sequence of the vaccine strains indicate putative N-linked glycosylation sites.

17 Boxes represent the ectodomains 1 and 2, and epitope B.

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		AA	AA	AA	AA	AA	AA	AA	AA	
		33	37	46	59	67	89	109	129	
		EPOXIDE						EPOXIDE		
VACCINE B	1	Sgqgnatlyqilvlllceagdrlnhgwvntfeyvathislgfittahfdalgvvetafgyryvceaypacfaafvfvraakomcrystftrfnlvndg								
VARI1	1K.....H.....								
VARI2	1K.....H.....								
VARI3	1K.....H.....								
VARI4	1K.....H.....								
VARI5	1K.....H.....								
VARI6	1K.....H.....								
VACCINE B	153	rikhwapiwklgaevgdv								
VARI1	153								
VARI2	153								
VARI3	153								
VARI4	153								
VARI5	153								
VARI6	153								
VACCINE A	1	Sgqgnatlyqilvlllceagdrlnhgwvntfeyvathislgfittahfdalgvvetafgyryvceaypacfaafvfvraakomcrystftrfnlvndg								
VARI7	1K.....H.....								
VARI8	1K.....H.....								
VARI9	1K.....H.....								
VARI10	1K.....H.....								
VACCINE A	153	rikhwapiwklgaevgdv								
VARI7	153								
VARI8	153								
VARI9	153								
VARI10	153								

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Strain	Origin	Date of sample collection/ year of submission	GenBank acc. number
HU01	lungs of an aborted fetus	20.06.2003.	DQ3666339
HU02	lungs of a dead fattening pig	20.10.2003.	DQ3666340
HU03	tonsil of a dead weaned pig	20.10.2004.	DQ3666341
HU04	lungs of a dead weaned pig	13.10.2003.	DQ3666342
HU05	lungs of a dead fattening pig	30.07.2004.	DQ3666343
HU06	lungs of a dead fattening pig	16.12.2004.	DQ3666344
HU07	lungs of a dead fattening pig	15.02.2005	DQ3666345
HU08	lungs of an aborted fetus	03.02.2005	DQ3666346
HU09	lungs of an aborted fetus	17.02.2005.	DQ3666347
HU10	lungs of a dead weaned pig	26.02.2005.	DQ3666348
HU11	lungs of an aborted fetus	06.04.2005.	DQ3666349
HU12	serum of a healthy weaned pig	11.05.2005.	DQ3666350
HU13*	healthy suckling piglet	25.10.2004.	DQ3666351
HU14	serum of a healthy gilt	25.10.2004.	DQ3666352
HU15	serum of an aborting sow	14.06.2004.	DQ3666353
HU16	lungs of a dead weaned pig	26.11.2004.	DQ3666354
HU17	serum of a weaned piglet	10.03.2005.	DQ3666355
HU18	lungs of a dead weaned pig	02.12.2004.	DQ3666356
HU19	lungs of a dead weaned pig	19.10.2004.	DQ3666357
HU20	lungs of a dead weaned pig	06.04.2005.	DQ3666358
HU21	lungs of a dead fattening pig	22.09.2006.	EF406336
HU22	lungs of an aborted fetus	11.09.2003.	EF406337
HU23	lungs of an aborted fetus	11.09.2003.	EF406338
HU24	lungs of an aborted fetus	18.09.2003.	EF406339
HU25	lungs of a dead fattening pig	20.10.2003.	EF406340
HU26	lungs of an aborted fetus	15.10.2004.	EF406341
HU27	lungs of a dead weaned pig	30.07.2004.	EF406342
HU28	lungs of a dead fattening pig	15.09.2004.	EF406343
HU29	lungs of a dead fattening pig	15.09.2004.	EF406344
HU30	lungs of a dead weaned pig	22.10.2004.	EF406345
HU31	lungs of a dead fattening pig	16.12.2004.	EF406346
HU32	lungs of a dead fattening pig	16.12.2004.	EF406347
HU33	lungs of a dead fattening pig	23.03.2005.	EF406348
HU34	lungs of a dead fattening pig	23.03.2005.	EF406349
HU35	lungs of a dead fattening pig	23.03.2005.	EF406350
HU36	lungs of a dead fattening pig	14.11.2005.	EF406351
HU37	lungs of a dead fattening pig	14.11.2005.	EF406352
AMERVAC	Spain	2006	DQ324668
BEL2001	Belgium	2001	AY035901
BOH2000	Czech Rep.	2000	AF253537
BRIT2002	England	2002	AF378799
DEN1992	Denmark	1998	AJ223078
DEN2001	Denmark	2001	AY035939
FRA1995	France	1996	U40697
ARNSBERG	Germany	2002	AF378797
ITA1995	Italy	1996	U40696
LELYSTAD	The Netherlands	1993	M96262
LITH01	Lithuania	2002	AF378800
LITH02	Lithuania	2002	AF378801
POL2002	Poland	2002	AF378804
PORCILIS	The Netherlands	2002	AF378819
PRIMEPAC	U.S.A.	1998	AF066384
PYRSVAC	Spain	2002	AF378820
QUE1994	Canada	1995	L40898
RESPPRRS	U.S.A.	1998	AF066183
SPA1996	Spain	1996	U40690
VR-2332	U.S.A.	1995	U87392
BEL-42	Belarus	2006	DQ324669
BOR-54	Belarus	2006	DQ324672
OBU-1	Belarus	2006	DQ324676
OKT-35	Belarus	2006	DQ324677
SNO-4	Belarus	2006	DQ324683
SOZ-6	Belarus	2006	DQ324686
VOS-49	Belarus	2006	DQ324690
ZAD-1	Belarus	2006	DQ324694
AUS	Lithuania	2006	DQ324667
SID	Lithuania	2006	DQ324682
FJ-1	China	2004	AY881994
GU992M	Japan	2005	AB175721
MD-001	Taiwan	1998	AF121131
01UD6	Thailand	2004	AY297113