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Full-length genome sequence analysis of a Hungarian Porcine Reproductive and Respiratory Syndrome Virus isolated from severe respiratory disease

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Abstract:	The authors report the isolation of a Type 1 PRRSV strain from a clinical outbreak of severe respiratory problems and high fever. Next generation sequencing was used to determine the complete genome sequence of the isolate (9625/2012). The virus belongs to a new branch within subtype 1, clade D, containing mostly Spanish sequences and shows highest similarity to PRRSV Olot/1991 and to the Amervac vaccine strain. SimPlot analysis performed with the available full-length genome sequences indicates no evidences of recombination. Mutation analysis of 9625/2012 and the most related strains revealed high proportion of amino acid substitutions in the putative neutralizing epitopes, suggesting an important role of the selective immune pressure in the evolution of PRRSV 9625/2012.
Response to Reviewers:	The manuscript by Balint et al reports the full genome sequence of an Hungarian isolate of Porcine Reproductive and Respiratory Syndrome virus (PRRSV) and phylogenetic analyses. Full genome sequences of PRRSV and studies on genetic diversity of PRRSV have been already reported; however, there is scarce complete genome data available for Hungarian PRRSV isolates so the report does provide some information that could be of interest to those investigators working in the field and

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	overall to readers of Archives of Virology. However, the study is more appropriate for a short report. The article has been shortened to a brief report format, but the overall structure has not been changed, the methods are concise and reproducible, the result and discussion sections are only shortened slightly. Specific comment are detail below: 1. Although the manuscript is well written, there are some inconsistencies in the abbreviations. For instance, page 3 line 36, it reads INF, the word should be spelled out completely the first time is used. It seems that the authors are refering to interferon and as such, this should be clear. If this is the case, the abbreviated form can be used in the discussion section, instead of the word "interferon." We have doublechecked the text for such inconsistencies, and the abbreviations are explained when necessary. 2. Phylogenetic trees should included an outgroup to obtain appropriate distances and to be more informative. We changed the phylogenetic tree by including acc. numbers, and marking our strain. Probably it was not highlighted enough but an outgroup (Type 2 reference strain VR2332) was already included in the original tree, and was also indicated in the text. We did not change that. 3. Some of the tables (primers, genbank accession etc) can be ommited. Genbank accession number information can be easily included in the phylogenetic tree. Genbank accession numbers were added to the phylogenetic tree, and contents of table 3 and 4 were added to the text. In our opinion the table descscibing the primers used for sequencing can not be omitted as it will be very confusing to list all the primer names, sequences and attachment sites in the text. Former Figure 1, showing SimPlot results has been omitted, as it shows no evidences of recombination, as it is basically a negative test, thus the results are described in the text.

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Full-length genome sequence analysis of a Hungarian Porcine Reproductive and Respiratory Syndrome Virus isolated from severe respiratory disease

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Keywords:
PRRSV, sequencing, complete genome, epitope

Abstract

The authors report the isolation of a Type 1 PRRSV strain from a clinical outbreak of severe respiratory problems and high fever. Next generation sequencing was used to determine the complete genome sequence of the isolate (9625/2012). The virus belongs to a new branch within subtype 1, clade D and shows the highest similarity to PRRSV Olot/1991 and to the Amervac vaccine strain. Mutation analysis of 9625/2012 indicates no evidences of recombination and it revealed high proportion of amino acid substitutions in the putative neutralizing epitopes, suggesting an important role of the selective immune pressure in the evolution of PRRSV 9625/2012.

Porcine Reproductive and Respiratory Syndrome (PRRS) is characterized by reproductive and respiratory symptoms, reductions in growth performance and increased mortality in young pigs. The etiological agent, Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) belongs to the Arterivirus genus within the *Arteriviridae* family [1], and its strains are divided into two major genotypes. Type 1 viruses are related to the early European isolates while Type 2 genotype viruses are related to the original North American strains. Recent data show that significant genetic variability can occur not only between but also within genotypes [2]. Excessive variability among Type 1 strains led to the introduction of four distinct subtypes [3].

The genetic variability may correspond to the wide range of symptoms extending from lack of clinical signs to fatal disease. Highly virulent PRRSV strains of both genotypes have been isolated from severe clinical cases from all over the world [4]. Type 1 subtype 3 strains 'Lena' and 'SU1-bel' induced increased clinical pathological effects in challenge models [5, 6].

Multiple genomic regions are responsible for the immunological behaviour and the virulence of the PRRSV and this fact underlines the significance of full-length genome sequencing. Several studies have confirmed the role of non-structural proteins of PRRSV in the modulation of host immune reactions mainly by blocking type I INF (interferon) responses of the innate immunity [7, 8]. It was shown that GP5, encoded by ORF5, contains B-cell neutralizing epitope, with the presence of multiple glycosylation sites and similarly to other Arteriviruses, an immunodominant decoy epitope is located in its proximity [9, 10]. T-cell epitopes have also been identified on both the GP5 and the N protein (encoded by ORF7) by *in silico* prediction and *ex vivo* evaluation [11].

Severe respiratory disease with high fever was observed in a non-vaccinated, originally PRRS-free fattening herd of 2500 animals (aged 4–5 months) in Eastern Hungary during 2012. The symptoms were resistant to antibiotic treatment, and in prolonged cases, posterior paresis and skin haemorrhages were observed. Despite the high (90%) morbidity, mortality remained at low level (0.2%). Thorough diagnostic examinations detected the presence of PRRSV in the clinical specimens originated from the infected herd.

The aim of the present study was to determine the full-length genome sequence of the PRRSV strain isolated from the aforementioned outbreak and to make an attempt to identify putative genetic markers contributing to the severe clinical disease, and to perform epidemiological investigations to reveal the origin of the strain.

Detailed diagnostic examinations of five perished pigs originating from the infected herds were performed, based on standard protocols of the National Food Chain Safety Office, Veterinary Diagnostic Directorate, Budapest, Hungary. For PRRSV detection, a real time PCR assay was executed as described earlier [12], whilst for sequencing of ORF5 and ORF7, primer pairs described by Balka et al. [13] were applied.

Major respiratory viral and bacterial swine pathogens like classical and African swine fever, SIV, ADV, PCV-2, *Actinobacillus pleuropneumoniae, Salmonella choleraesuis, Streptococcus suis* were tested according to the current protocols of the World Organisation for Animal Health [14]. *Mycoplasma hyopneumoniae* was tested by PCR, according to Stemke et al. [15].

Culture of porcine alveolar macrophages (PAMs) was prepared according to the OIE Manuals [14]. Lung tissue homogenates of PRRSV 9625/2012 were prepared with Tissue Lyser (Qiagen, Hilden, Germany) in sterile phosphate-buffered saline (PBS) containing antibiotics and antimycotics, and centrifuged at 5000 ×g for 10 min. Cell-free supernatants were aliquoted and stored at –80°C for inoculation and RNA isolation. PAMs were inoculated with 100 µl of supernatant and incubated for five days.

RNA was isolated with QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and cDNA was generated using Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) kit and a T_{20} primer, according to the protocol of the manufacturer.

The genome of PRRSV 9625/2012 was amplified in six overlapping parts, using The Phusion II HotStart PCR kit (Thermo Scientific, Waltham, MA, USA), in 25 µl final volume with 1 µl of cDNA template, in 1x GC buffer in the presence of 4% DMSO. Amplification was performed using the following gradient PCR programme: 98°C 1', 35 × [98°C 20", 54-72°C (gradient ramp: 2°C) 20", 72°C 5'], 72°C 5'. The primer pairs used for the amplification of the different fragments are listed in Table 1. For determination of both ends of the genome, the 5′ RACE System for Rapid Amplification of cDNA Ends, version 2.0 (Invitrogen, Carlsbad, CA, USA) as well as a forward ORF7 primer [13] and the T_{20} primer were applied.

An equimolar mixture of the overlapping PRRSV PCR products was used as template for next generation sequencing. Sequencing was carried out on a 316 Chip using the Ion Torrent semiconductor sequencing equipment (Ion Personal Genome Machine® (PGMTM); Life Technologies) according to the manufacturer's recommendation. Sequences were assembled and aligned with the SeqMan Ngen software (Lasergene, Madison, WI, USA).

MEGA software v.5.1 [16] was used to align DNA and protein sequences and to build a phylogenetic reconstruction. Neighbor-joining algorithm was implemented with the Kimura-2 parameters model using a transition-to-translation ratio of 2.0. The topology of trees was confirmed by 1000 bootstrap replicates. The SimPlot programme [17] was applied for visualizing similarity along the whole genome. Potential N-linked glycosylation sites (score >0.5) were predicted by the NetNGlyc 1.0 Server [18].

The presence of PRRSV and *Mycoplasma hyopneumoniae* in the five investigated pigs was confirmed by real-time PCR and conventional PCR, respectively. The absence of other respiratory and generalized bacterial infection was diagnostically confirmed by bacterial cultivation, serology, and PCR.

The full length sequence of PRRSV 9625/2012 was 15098 nt long (Genbank Acc. No: KJ415276). At genome level, the overall nucleotide identity was 96%, 95%, 92% and 58% with PRRSV strains Amervac MLV, Olot/1991, Lelystad (acc.: M96262) and the Type 2 prototype strain VR-2332 (acc.: U87392), respectively.

PRRSV 9625/2012 showed the closest similarity to the vaccine strain Amervac MLV (613 nucleotide change with no deletion/insertion) raising the possibility of direct descendancy between the two viruses, which could have been facilitated by viral transmission after vaccination.

No signs of recombination with Amervac MLV or other PRRSV strains were observed according to the SimPlot analysis (data not shown) indicating the lack of such an event in the evolution of PRRSV 9625/2012. Throughout the whole genome the Amervac MLV and Olot/91 strains exhibited the closest similarity, ranging between 86–99%, but never reached 100%. Nucleotide similarity fluctuated between 79–98% and 0–73% with PRRSV type 1 Lelystad and Type 2 VR-2332 reference strains, respectively.

The phylogenetic tree using ORF5 sequences from a selection of Type 1 strains and VR-2332 as outgroup revealed that PRRSV 9625/2012 is a Type 1, subtype 1 virus within Clade D, defined by Shi et al. 2010 [5]. The closest (96%) nucleotide identity was shared with Spanish and other, MLV-like strains (Fig. 1) that have already been present in Hungary for a decade [13], but our strain seems to represent a previously unknown evolutionary branch within this clade.

The analysis of the putative amino acid (aa) sequences of the aforementioned three strains revealed that the most aa variations are found in Nsp1 (93% identity, 96–97%

similarity) and Nsp2 (93% identity, 94–95% similarity). The structural proteins of PRRSV 9625/2012 showed 94–98% identity with Amervac MLV and Olot/1991.

We compared the antigenic regions (ARs) within GP2, GP3, GP4 and GP5 proteins of PRRSV 9625/2012 with Amervac MLV and Olot/1991, since these proteins are the main targets for neutralizing antibodies.

In the two neutralizing epitopes AR II and AR IV of GP2 [9, 19], one-one aa substitutions were found, respectively (Fig. 2a). Additionally, two aa substitutions were also detected right upstream of AR II. In the non-neutralizing ARs I, III and V, two, one and zero aa substitutions were observed, respectively. In the non-antigenic part of the protein, only three aa differences were found (Fig 2a).

In the two neutralizing epitopes AR I and AR VIII [19, 20] of GP3, two and three aa substitutions were found, respectively, whilst the non-neutralizing AR II contained two mutations (Fig. 2b). The non-antigenic part of the protein contained five aa substitutions.

Four aa substitutions were found in the neutralizing epitope AR I of GP4 [19, 21], while three mutations were observed in the non-immunogenic part of the protein (Fig. 2c).

In the neutralizing epitope of Type I PRRSV GP5 [22], one aa substitution was found. AR I, the main epitope targeted by neutralizing antibodies in Type II strains [10], but non-neutralizing epitope in the case of Type I PRRSV [19, 23], demonstrated a single aa substitution. The other non-neutralizing epitopes of GP5, AR II and AR IV contain no aa mutations. The non-antigenic region of the protein contained four aa substitutions (Fig. 2d). It is striking that a disproportionally large number of the aa changes in ORF2-4 occur in known B cell epitopes which suggests a strong immunological pressure in these regions.

The GP5 of isolate 9625/2012 contained three putative N-glycosylation sites at aa 37, 46 and 53. That of PRRSV Amervac MLV also contained three at 35, 46 and 53, while GP5 of Olot/1991 had only two putative N-glycosylation sites at aa 46 and 53.

Both PRRSV genotypes change at a rate of 0.5–1% per year in the field [24]. The 96% similarity observed between 9625/2012 and Olot/1991/Amervac MLV may equate to 4–8 years of changing in field. Since 2004, strains with 98–99% similarity to the vaccine strain have been identified in herds vaccinated with Amervac MLV [13, Bálint, personal data]. However, PRRSV 9625/2012 had higher genetic distance from the vaccine strain, and occurred in a fattener farm that has never been vaccinated against PRRS. Yet the most plausible explanation of the origin of PRRSV 9625/2012 is that it descended from Amervac MLV or an Olot/1991-like, unidentified strain between 2004 and 2008, and it circulated/evolved without being identified in Hungary until its appearance in 2012. Another explanation could be that it was newly introduced to Hungary in 2012 as a new PRRSV variant from an unidentified source.

Analysis of the putative amino acid sequence revealed the presence of relatively high 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 IFN synthesis and signalling pathway [25]. Furthermore, nsp2 is the viral protein containing the highest frequency in B cell epitopes [26]. The amino acid sequence variability in this protein is high both within and between genotypes [27], and changes including deletions in these parts of the genome might have an influence on the pathogenicity of the strains [10].

The two aa substitutions in the neutralizing epitopes and the three aa differences in the non-neutralizing ARs, versus the three aa substitutions in the non-antigenic part of the GP2 protein suggest the role of selective immune pressure in the development of this strain. Those variants that show bigger differences on the antigenic regions compared to the original strains might have the ability to avoid or decrease the efficacy of neutralizing antibody attachment. Similar tendencies have already been identified in PRRSV circulating in Hungary [13]. The two aa substitutions right upstream of AR II suggest that this region might be extended N-

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65

terminally, considering that its terminal border was mapped originally with a precision of about two amino acids [9].

It has been long suspected that an 'epitope site' covering ARs GP3-I and GP3-II is involved in functional interactions with the host cell [9, 28] but it was only recently proven that antibodies against AR I have neutralizing capacity [19]. The three aa substitutions in AR I and AR II, as well as the three found in the neutralizing epitope of AR VIII, may also reflect immunogenic pressure.

In GP4, a neutralizing epitope is present in Type I PRRSV from aa 57 to 68 [21] within AR I [19]. The three aa substitutions found within this sequence confirm that this region is under antibody-mediated pressure *in vitro* and *in vivo* [29]. Whether these substitutions contribute to the generation of an escape mutant that is not cross-neutralized with PRRSV Amervac MLV or Olot/1991 is still unknown.

GP5 has been considered to be the main target for virus neutralizing antibodies in North American PRRSV strains [10]. A neutralizing epitope has been identified at positions 37–45 [10], but this epitope proved to be non-neutralizing in the case of Type I PRRSV [19, 23]. The neutralizing epitope in Type I GP5 is located upstream of this epitope from residues 29 to 35 [22]. The three amino-acid substitutions present from aa 36–38 spanning the two aforementioned epitopes may suggest a role of selective immunological pressure in the sequence change.

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 [30]. Moreover, increased production of proinflammatory cytokines have been observed following dual inoculation with these pathogens when compared to single infections [31].

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Table and Figure legends

Table 1. Oligonucleotide primers used for RT-PCR amplification of PRRSV 9625/2012.

Figure 1. Phlyogenetic analysis of the ORF5 coding region of PRRSV 9625/2012 using the neighbour-joining method and adding VR-2332 Type 2 reference strain as outgroup. The topology of the tree was confirmed by 1000 bootstrapping steps.

Figure 2. Alignment of a, ORF2; b, ORF3; c, ORF4 and d, ORF5. Antigenic regions are shaded. Underlined sequences represent neutralizing epitopes.

Figure

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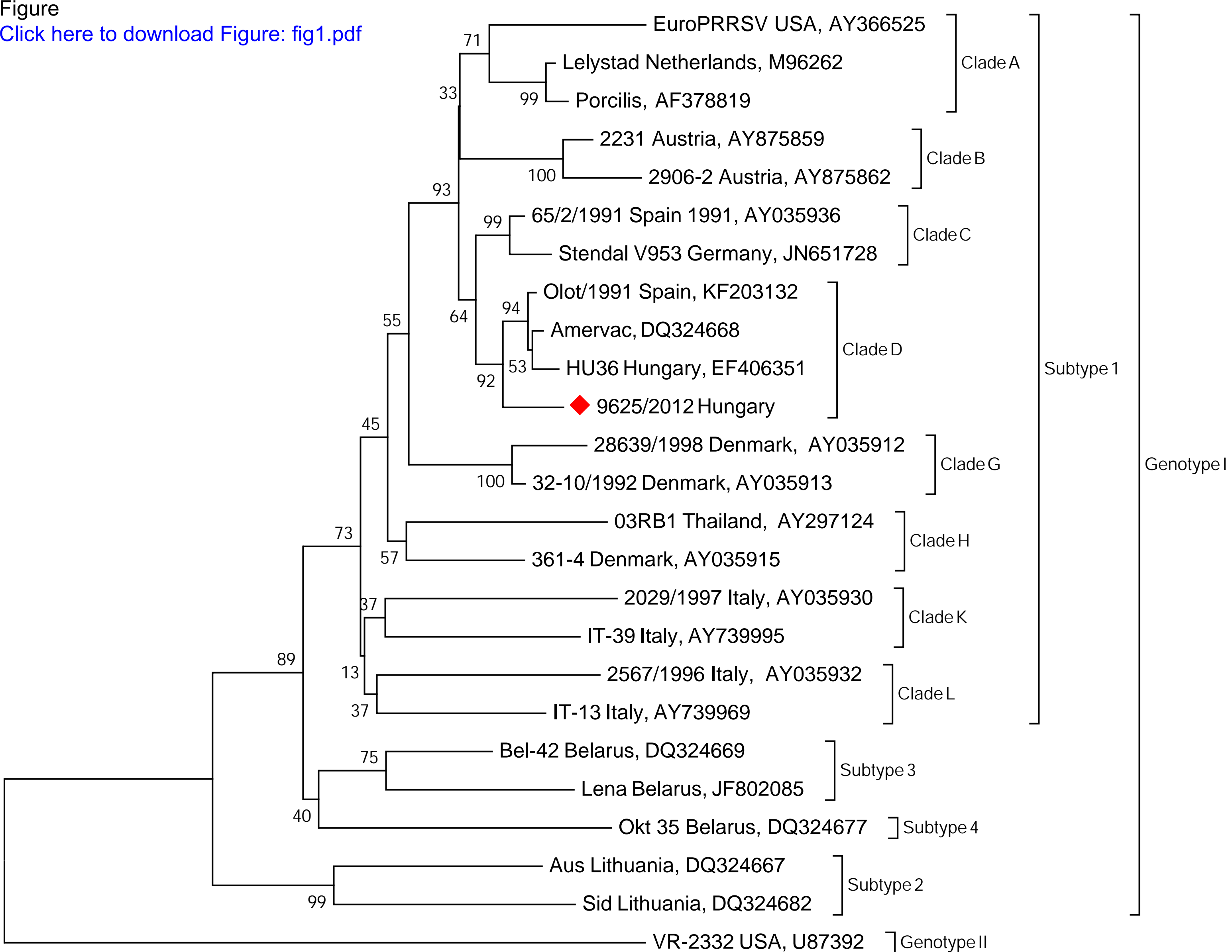

Figure
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Figure 2. Alignment of a, ORF2; b, ORF3; c, ORF4 and d, ORF5. Antigenic regions are shaded. Underlined sequences represent neutralizing epitopes.

a,

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                 AR I                               AR II                                                         AR III 100
9625/2012  : MQWGHCGVKSASCSWTPSLSSLLVWLTLSFFSPYCSGSPSQDGYWSFFSEWFAPRFSVRALPFTLPNYRRSYEGLLPNCRPDVPQFAFKHPLGMFWHMRV
AMERVAC    : ....Y....L..............I...SL...L.........................................S..............IL.....
OLOT/1991  : ....Y....L..............I...SL...L..........S..............................S..............IL.....

                              AR IV                                                                                  200
9625/2012  : SHLIDEMVSRRIYRTMEHSGQAAWKQVVSEATLTKLSGLDIVTHFQHLAAVEADSCRFLSSRLVMLKNLAVGNVSLQYNTTLDRVELIFPTPGTRPKLTD
AMERVAC    : ...................S.......................................................H................
OLOT/1991  : ...................S.......................................................H................

                AR V                           240
9625/2012  : FRQWLVSVHASIFSSVASSVTLFIVFWLRIPAVRYVFGFHWPTATHHSS
AMERVAC    : .................................................
OLOT/1991  : ................................X................
```

b,

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                                                                AR I                AR II                    100
9625/2012  : MARQCTRFHFFLCGFICYLVHSVLATNSSRTLCFWFPLAHGNTSFELTINYTICMPCPTSQAAKQRLEPGRNMWCRIGQDRCEERDHDELSMSIPSGYEN
AMERVAC    : ..H..A...................S..........................S.....S.....................H.....................
OLOT/1991  : ..H..A...................S...X......................S.....S.....................H.....................

                                                                                                             200
9625/2012  : LKLEGYYAWLAFLSFSYAAQSHPELFGIGNVSRVFVDKRHQFICAEHDGQNSTISTEHNISASYAVYYHHQIDGGNWFHLEWLRPFFSSWLVLNISWFLR
AMERVAC    : ....................F...................................G.....L.....................................
OLOT/1991  : ....................F...................................G.....L.....................................

                                                           AR VIII   260
9625/2012  : RSPVSPVSRRIYQILRPTRPRLPVSWSFRTSIVSDLTGSQQRKRLFPSENRLNVARPSAFPSTSR
AMERVAC    : ............................................P....S.P......V....L.
OLOT/1991  : ............................................P....S.P......V....L.
```

c,

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                                                                          AR I                                 100
9625/2012   : MAAAILFLLVGAQYLMVSEAFACKPCFSTHLSDIKTNTTAAAGFLVLQDINCLRPHGVPTAQETLSIRKPSQCREAVGIPQYITITANVTDESYLYNADL
AMERVAC     : .........A..................................M.............S.....I.FG................................
OLOT/1991   : .........A..................................M.............S.....I.FG................................

                                                                                          180
9625/2012   : LMLSACLFYASEMSEKGFKVIFGNVSGVVSACVNFTDYVAHVIQHTQQHHLAIDHIRLLHFLTPSTMRWATTIACLFAILLAI
AMERVAC     : ...................................................V...............................
OLOT/1991   : ...................................................V...............................
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d,

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                                                   AR I                                                        100
9625/2012   : MRCSHKLGRFLTLHSCFWWLFLLCTGLSWSFVDGSDNSSTYQYIYNLTICELNGTDWLSSHFDWAVETFVLYPVATHILSLGFLTTSHFFDALGLGAVST
AMERVAC     : .......E....P......................N.S..................E..P.........................................
OLOT/1991   : .......E....P......................NGB..................E..P.........................................

                                                             AR II             AR III                   AR IV  200
9625/2012   : TGFIGGRYVLSSVYGACAFAALVCFVIRAAKNCMACRYARTRFTNFIVDDRGRIHRWKSPIVVEKLGKAEVGGDLVTIKHVVLEGVKAQPLTRTSAEQWEA
AMERVAC     : ...V.................................................................................................
OLOT/1991   : ...V.................................................V...............................................
```

Table

Table 1. Oligonucleotide primers used for RT-PCR amplification of PRRSV 9625/2012

Primer	Sequence	Position
PR_EU_15F	ATTCCCCCTACATACACGACAC	15-36
PR_AVAC_2477F	CTCGGACTCCATGAAAGGAA	2477-2496
PR_EU_4719F	CCATGGTGTTCAAATCCTTTTT	4719-4740
PR_AVAC_9300F	TGTGGGAGAAACTGAAAAGTCA	9300-9321
PR_AVAC_11818F	TAAAATTAGCCAGCTGTTCGTG	11818-11839
PR_AVAC_2902R	ATGATAGAGCTGGATTCGGAAA	2902-2881
PR_AVAC_5337R	CGGTGTTAAGGCAGGGTTTATG	5337-5316
PR_AVAC_9859R	CATAGTCTGATGGGTGGGTGTA	9859-9838
PR_AVAC_12552R	TTGATGGTTAGCTCGAATGATG	12552-12531
Pr15047R	T(24)AATTTCGGTCACATGGTTCCTGCCTG	15098-15073