Genetic and immunobiological diversity of porcine reproductive and respiratory syndrome genotype I strains

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Summary

Genetic diversity of porcine reproductive and respiratory syndrome virus (PRRSV) has been based on ORF5/GP5 and ORF7/N protein variations. Complete viral genome studies are limited and focused on a single or a few set of strains. Moreover, there is a general tendency to extrapolate results obtained from a single isolate to the overall PRRSV population. In the present study, six genotype-I isolates of PRRSV were sequenced from ORF1a to ORF7. Phylogenetic comparisons and the variability degree of known linear B-epitopes were done considering other available full-length genotype-I sequences. Cytokine induction of all strains was also evaluated in different cellular systems. Non structural protein 2 (nsp2) was the most variable part of the virus with 2 out of 6 strains harboring a 74 aa deletion. Deletions were also found in ORF3 and ORF4. Phylogenetic analyses showed that isolates could be grouped differently depending on the ORF examined and the highest similarity with the full genome cluster was found for the nsp9. Interestingly, most of predicted linear B-epitopes in the literature, particularly in nsp2 and GP4 regions, were found deleted or varied in some of our isolates. Moreover, 4 strains, those with deletions in nsp2, induced TNF-α and 3 induced IL-10. These results underline the high genetic diversity of PRRSV mainly in nsp1, nsp2 and ORFs 3 and 4. This variability also affects most of the known linear B-epitopes of the virus. Accordingly, different PRRSV strains might have substantially different immunobiological properties. These data can contribute to the understanding of PRRSV complexity.

Keywords: Porcine reproductive and respiratory syndrome virus; complete ORFs sequences; genotype-I; phylogenetic analyses; immunobiological properties.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) emerged in the last years of the decade of 1980s and by early 1990s became one of the major pathogens affecting the swine industry. At present, some 20 years after its emergence (Keffaber., 1989; Paton et al., 1991), PRRSV and the syndrome that it causes are still little understood and fully efficacious vaccines are lacking. The reasons for such a lack of vaccines are diverse but the high genetic diversity of the virus and the ability of PRRSV for subverting the immune response of the host are paramount. PRRSV is a positive-sense ssRNA enveloped virus classified within the genus *Arterivirus*. Nowadays, two genotypes are recognized (I and II) that originally were described as European and American because of the geographic origin of their prototypic strains (Lelystad virus and VR-2332), respectively (Collins et al., 1992; Wensvoort et al., 1991). Genetic diversity within genotype-I PRRSV isolates is high (Forsberg et al., 2002; Mateu et al., 2003; Stadejek et al., 2002) and at least three or four subtypes are being recognized (Stadejek et al., 2002, 2006, 2008).

PRRSV genome is organized in 9 open reading frames (ORF). ORF1a and 1b encode the viral replicase as well as other non structural proteins (nsp) (Snijder and Meulenberg., 1998).Of these nsp, nsp2 is the largest and has been shown to contain a large cluster of B-epitopes (Oleksiewicz et al., 2001). Recently, nsp2 and nsp1 of genotype II strains have been reported to be involved in the interplay between the virus and the host response (Beura et al., 2010; Chen et al., 2010, Subramaniam et al., 2010) by down-regulating mediation of interferons and other cytokines. ORFs 2a, 2b and 3-7 encode for the viral structural proteins. Most of the abovementioned studies about

genetic diversity of PRRSV have been developed by examining ORF5 or ORF7 but also some papers focused on ORF3 which is thought to be a molecular clock for PRRSV evolution (Forsberg et al., 2001). Surprisingly, a GenBank search for full length genome –or almost complete genome- PRRSV sequences of genotype I revealed the scarcity of these data and less than 10 full sequences could be found. The aim of the present study was to produce and analyze the sequence of ORFs 1a to 7 of contemporary PRRSV field strains of genotype-I for determining within genome diversity and phylogenetic relatedness and to examine some of the immunobiological properties associated with those strains.

Material and methods

. Strains and sequencing. Six field PRRSV strains (2982, 3249, 3256, 3262, 3266 and, 3267) were randomly selected among non epidemiological related strains. Additionally, the vaccine strain Porcilis PRRS was also sequenced. Origin and characteristics of these strains are shown in Table 1. PRRSV strains were grown in porcine alveolar macrophages (PAM) obtained from high health pigs of a farm historically free from all major pig diseases including pseudorabies, classical swine fever, PRRS and influenza. For assessing purity of the viral production, PAM batches were tested by reverse transcription (RT)-PCR or PCR for PRRSV, hepatitis E virus, Torque tenovirus and porcine circovirus type 2 according to previously published protocols (Martin et al., 2007; Mateu et al., 2003; Quintana et al., 2002; Segalés et al., 2009). Viral batches were tested for all those pathogens as well. All viral isolates were used as PAM supernatants (passage n=3). Viral RNA was extracted from supernatants by using the QiaAMP viral RNA minikit (Qiagen, Barcelona, Spain) according to the manufacturer instructions.

Reverse transcription was done using Superscript II Reverse transcriptase and random hexamers (Invitrogen, Barcelona, Spain). The obtained cDNA was then used in specific PCRs designed to amplify overlapping segments of the viral genome (Table 2). Both strands of PCR products were sequenced using the Genetic Analyzer 3130 XL (Applied Biosystems). When deletions in the genome were found, confirmation was carried out by re-sequencing of the products and synthesizing of new primers flanking the supposed deletion and further amplification and sequencing.

. Analysis of viral sequences. Sequences were examined and purged of errors using Chromas Pro 1 (Larkin et al., 2007). Predicted amino acid sequences were obtained by translation using BioEdit (Hall., 1999). Nucleotide sequences were aligned using Clustal X2 with correction for multiple substitutions. Bootstrap values were calculated after 1000 iterations using the Neighbor-joining method. For comparative purposes available full length sequences of genotype I (Genbank Accession numbers: M96262-Lelystad virus; AY366525-North American EuroPRRSV; AY588319-LV-4.2.1; DQ864705-strain 01CB1 from Thailand; DQ489311-viral clone; EU076704-strain HKEU16 from Hong Kong; FJ349261- strain KNU07 from Korea and GQ461593-strain SHE from China) and genotype II (Genbank accession numbers: AF066183, AF325691, AY032626, AY424271, AY545985, G EU880437, EU708726, Q857656, NC_001961, U87392) PRRSV sequences were also included. Synonymous (dS) and non-synonymous (dNS) substitutions in each ORF were calculated using SNAP (Körber., 2000) available at http://hcv.lanl.gov/content/sequence/SNAP/SNAP.html and expressed as dS-dNS values. Topological trees were built up with Mega4 (Tamura et al., 2007) and rooted on mid-points. Predicted amino acid sequences were also examined for variations in the linear B-epitopes reported by Oleksiewicz et al. (2000,

2001), and in the neutralization epitopes (NE) known to be located in GP4 (Meulenberg et al., 1997) and GP5 (Plagemann., 2004).

. Cytokine induction in peripheral blood CD172a$^+$ cells and PAM. Recent papers indicated the role of non structural proteins in the regulation of the immune response to PRRSV through cytokine release. Since full ORF1a and 1b sequences were available, cytokine induction by the different PRRSV isolates was examined using peripheral blood CD172a$^+$ cells or alveolar macrophages. PBMC and PAMs were obtained from healthy pigs of the same litters than above. CD172a$^+$ cells were purified from PBMC by positive selection using MACS Microbeads (Miltenyi Biotech SL, Spain). Briefly, cells were incubated with mouse anti porcine CD172a-FITC (Serotec, Spain) on ice for 30 minutes. After incubation, PBMC were washed and CD172a$^+$ cells were coupled (15 min on ice) with anti-FITC magnetic particles (Miltenyi Biotech SL, Spain). PBMC were washed again, re-suspended in MACS buffer (PBS plus foetal calf serum) and labeled cells were retrieved using LS selection columns (Miltenyi Biotec SL, Spain) according to manufacturer's instructions. Purity of the cellular suspension was examined by flow cytometry analysis before further characterization. The obtained cell suspension always had a richness of CD172a$^+$ $\geq$ 92%. PAM were obtained by bronchoalveolar lavage of lungs of piglets. Briefly, after humane euthanasia, lungs were removed aseptically and washed by infusion through the trachea of PBS (Sigma, Spain) supplemented with 2% gentamicin. The retrieved cell suspension was centrifuged (10 minutes at 450 g), washed and cells were frozen at -150°C until needed. PAM were produced by adhesion to plastic of the retrieved cells. Parallel cultures of PAM were always examined for absence of pathogens as described above. PAM and CD172a+ cells were seeded at a density of $5x10^5$ cells/well in 0.5 ml volume and stimulated with viable PRRSV (2982; 3249; 3256; 3262; 3266 and 3267 strains) at 0.01 m.o.i for 24 h.

All strains were examined three times (separate days), in triplicate cultures each time. For a given series of tests, all strains were tested in cells coming from the same animals. As a negative control, supernatants from mock-infected PAM were included. Each time, cell culture supernatants of the three replicas were collected and mixed and the resulting mixtures were examined by ELISA for determining concentrations of different cytokines. Because of the greater sensitivity of CD172a+ for producing IFN-α, IL-10 and TNF-α **(**Gimeno et al., in press), these cytokines were examined in cell culture supernatants of $CD172a^+$ cells while supernatants of PAM were used to examine IL-1, IL-6 and IL-8. IFN-α capture ELISA was performed as reported previously (Guzylack-Piriou et al., 2004) using K9 and F17 monoclonal antibodies (Grupo Taper SA, Madrid, Spain). F17 was biotinylated (Phase Biotinylation Kit, PIERCE, Spain). IFN-α recombinated protein (PBL Biomedical lab, Piscataway, New Jersey) was used as a standard. IL-10 capture ELISA was performed using commercial pairs of mAbs (swine IL-10, Invitrogen, Barcelona, Spain) (Díaz et al., 2005). TNF-α, IL-1, IL-6 and IL-8 capture ELISAs were performed using matched antibody pairs according to manufacturer's instructions (R&D systems, Spain). The cut-off point of each ELISA was calculated as the mean optical density of negative controls plus three standard deviations. Values for the cytokine concentration in cell culture supernatants were calculated as a corrected concentration resulting of the subtraction of cytokine levels in mock-stimulated cultures from the values obtained for virus-stimulated cultures (concentration $_{PRRSV}$-concentration $_{mock}$).

.**Statistics.** Statistical analysis was done using StatsDirect 2.7.5. χ^2 test was used to compare proportion of substitutions in the different viral proteins; comparison of results obtained in VNT and ELISPOT was done using the Kruskal-Wallis test.

Results

. Alignment and comparison of PRRSV genomic and predicted amino acid (aa) sequences. Global results for the comparison of ORFs and predicted proteins are shown in Table 3. Average similarity of the different ORFs among genotype I strains ranged from 94.7%±2.4% for ORF7 to 89.5%±4.4% for ORF1a. However this value for ORF1a was strongly biased by the different values of similarity in each of the ORF1a-encoded nsp. Thus, the higher variation was found in nsp2 which average similarity among examined genotype I strains (aa) was only of 83.3%±6.7% followed by nsp1αβ with a 88.4%±4.5%. Interestingly, most of the variation in nsp1 accumulated in nsp1β where between aa positions 180 and 305 up to 66 substitutions were found (53% of the positions). Figure 1 shows the aa alignment for nsp1. In nsp2, variation was even higher and concentrated between aa positions 269 and 430 with up to 88 positions with substitutions (55%). However, the most relevant finding was the existence of deletions of different length. Strains 2982 and 3262 had a 74 aa deletion (positions 308-381), longer than any previously known nsp2 deletion in genotype I strains, for example AY366525, DQ489311 or FJ349261 (Figure 2). Strain 3256 had a 25 aa deletion distributed in two non-contiguous segments (346-358 and 418-429) and strain 3249 has a 1 aa deletion (aa 349). Porcilis PRRS also had a 74 aa deletion starting from position 281. When nsp2 protein sequences were compared among genotype I and II PRRSV strains it was found that the percentage of similarity was the lowest of all viral proteins (26.3%). Similarities below 50% in the aa sequence were also found for nsp7 and nsp12. Average similarities for other proteins encoded by ORF1a or ORF1b were well above 90%.

Examination of the other ORFs and of the corresponding predicted proteins showed that ORF3 and ORF4 were the next genes with a higher variability (89.9% of average similarity in both cases); however, the percentage of positions with amino acid substitutions showed a trend for being higher in GP4 (57 out of 183 aa, 31%) compared to GP3 (63 out of 265 aa, 24%; p=0.08). Figures 3 and 4 show the alignments for GP3 and GP4, respectively. For GP3 a two aa deletion was found in strain 3262 (positions 235-236) corresponding to the same region previously reported to be deleted for Hong Kong strain EU076704. In GP4, a two aa deletion was also found in strain 3262 (positions 59-60) upstream of a known eight aa deletion in strain EU076704 (positions 57-64). Regarding GP5, it is worth noting that 30% of the aa positions could harbor a substitution being the fourth viral protein after nsp2, nsp1 and GP4 in the proportion of variable positions. The global analysis of dS-dNS substitutions showed that density of dNS mutations was higher in nsp1, nsp2 , the carboxy-terminal segment of GP3, the whole GP4 and the amino-terminal part of GP5 (Figure 5).

. Phylogenetic grouping of PRRSV strains. Phylogenetic analysis of whole ORFs 1-7 is shown in figure 6. According to the bootstrapped phylogenetic tree, strains 2982 and 3262 formed a cluster; strain 3266 clustered with DQ864705; 3267 clustered with GQ461593 and strains 3249 and 3256 grouped together. When phylogenetic trees were calculated based on the particular ORF sequences of those same strains, it was observed that grouping of sequences varied depending on the ORF or even the segment used. For example, in ORF4 strain 2982 formed a significant cluster with strain GQ461593 and strain 3267; in contrast to ORFs 1a and 1b where 2982 clustered with 3262. In ORF5 strain 3249 clustered with FJ349261 but did not in any other ORF, and 3249 clustered with 3267 in nsp5 (not shown). Globally, grouping obtained with ORF1b was the most similar to that obtained with the whole virus sequence. In contrast, grouping based on

ORF5 or ORF7 produce very dissimilar trees when compared with the one obtained with the almost whole viral sequence (Figure 6). Since sequencing of whole ORF1b is not useful for practical purposes, trees based on individual nsp were examined. Of these, nsp9 results were the most similar to the whole virus examination (Figure 6).

. Variation in linear B-epitopes of PRRSV. Predicted amino acid sequences were examined for changes in B-epitopes reported before in the literature. Regarding the known linear epitopes described by Oleksiewicz *et al.* (2001) it is worth to note that out of nine serologically confirmed epitopes, seven (ES1, ES3, ES4, ES6, ES7, ES9, ES11) had substantial variations. Particularly, ES3 and ES4 were deleted in strains 2982 and 3262. Figures 1 and 2 show variations in the cluster of epitopes present in nsp1 and nsp2. Regarding the epitope reported by Oleksiewicz *et al.* (2000) in GP3 (which core was RKASLSTS), that epitope was absent in the examined sequences. Variations in the core of the NE in GP4 (aa 59-66 in Lelystad virus) were found in most of the sequences; deletions were even found in strains 3262 and EU076704 (Figure 4). In contrast, for the NE in GP5, variations were only found regarding the glycosylation site at N-37 and thus, strains 2982, 3256 and 3262 were predicted to have this third glycosylation site at N-37 (not shown).

. Cytokine induction by different strains. Table 4 shows the results of the cytokine ELISAs. All tested strains were unable to induce IFN-α or IL-6 release; in contrast, all of them induced very high levels of IL-8 and IL-1 and, particularly, strain 3256 induced the highest IL-1 levels. Regarding TNF-α, only strains 2982, 3249, 3256 and, 3262 induced release of this cytokine, being 3262 the strongest inducer. Strains 2982, 3256 and 3262 induced secretion of IL-10, being the 3256 the one yielding the higher response.

Discussion

A review of the literature about the genetic diversity and variations of genotype-I PRRSV reveals that most of the papers deal with variations in ORF5/GP5 and ORF7/N protein. The scarce remaining papers that examined the whole viral genome or almost the whole viral genome (Amonsin et al., 2009; Fang et al., 2004; Nam et al., 2009; Roop et al., 2004) were mostly focused in a particular strain or a very narrow set of strains. Therefore, knowledge in that area is limited and often this has leaded to assume that most facts described for a single genotype-I or genotype-II isolate could represent all PRRSV strains.

The present study further confirms that diversity of genotype-I isolates of PRRSV is high and, that variability within the viral genome is unequally distributed. Thus, ORF1a, ORF3 and ORF4 account for the highest diversity (10.5%; 10.1% and 10.1%, of variation, respectively). The examination of the topological distribution of dS-dNS offers a finer picture of this. Within ORF1a, most substitutions and deletions accumulate in nsp1 and nsp2 while for other nsp in that ORF1a, similarities are over 95%. Within nsp1, the highest variability is observed in nsp1β. Recently, Beura *et al.* (2010) showed the importance of nsp1 for inhibiting activation of the IFN-β promoter and that nsp1β inhibited NF-κβ signalling. According to those authors, IL-8 mRNA induction upon dsRNA treatment (mediated by TLR-3) was reduced by nsp1β expression. In our study, in spite of the extremely high variability found in nsp1β (up to 98 positions with substitutions out of 219 aa; 45%) none of the six tested strains had the ability to induce detectable levels of IFN-α and, contrarily to what could be expected from the results of Beura *et al.* (2010), all strains were extremely able inducers of IL-8,

suggesting either that our system (PAM) is not compatible to HEK-293 cells used by Beura *et al.*, that other parts of the virus may also be good IL-8 inducers, or that levels of nsp1β necessary to produce such an inhibition of IL-8 mRNA are attainable in nsp1β -transfected HEK-293 cells but not in infected PAM.

Nevertheless, the most variable part of the virus was nsp2. In the present study the length of the predicted protein varied from 861 aa (i.e. Lelystad virus) to 787 aa in strains 2982 and 3262. The 74 aa deletion in nsp2 of those two isolates is the largest deletion known up to date in genotype-I strains (Nam et al., 2009; Roop et al., 2004) and confirm the non-essential nature of the segment comprised between residues 280 and 380 of genotype-I nsp2. Moreover, it is interesting to note that the most negative dS-dNS values were located in nsp2 and that similarity between genotype-I nsp2 and genotype-II nsp2 can be as low as 24%. These facts evidence that evolutive pressures act with high intensity upon this protein and are compatible with an important role of this protein in the immune regulation. Chen *et al.* (2010) suggested recently that deletion of some epitopes in nsp2 may lead to substantial changes in the biological properties of a given PRRSV strain. According to those authors, deletion of epitope ES3 in nsp2 probably result in an enhanced *in vitro* an *in vivo* replicative ability as well as decreased ability to induce IL-1β and TNF-α responses in peripheral blood mononuclear cells or macrophages. In the case of the present study, strains 2982 and 3262 that had natural deletion of ES3 did not replicate better than strains harbouring a full nsp2 (i.e. 3266 or 3267) even they yielded lower titres. However, since parental – undeleted- ancestor of strains 2982 or 3262 were unknown, it cannot be ascertained if the deletion had or not an essential effect on the replicative ability of PRRSV in macrophages but it is interesting to remark that the vaccine strain Porcilis PRRS, that is

known for replicating poorly in macrophages, also harboured a 74 aa deletion in that segment of nsp2.

Regarding the cytokine induction by the examined strains, it is worth to remark that strains with extensive 25 aa or 74 aa deletions in nsp2 were the only inducing simultaneously IL-10 and TNF-α release. In the case of 3262 (with a 74 aa deletion affecting ES3 and ES4), levels of TNF-α were the highest of the six examined strains. These results are in apparent contradiction with those reported by Chen *et al.* (2010) who observed that deletion in ES3 seemed to impaired TNF-α release. As seen in the present paper, field strains are in fact diverse and may harbour different deletions and substantial variations in nsp2. Other authors have also reported that nsp1 suppresses tumor necrosis factor-alpha promoter activation (Subramaniam et al., 2010). In this scenario, the biological properties of a given strain will be the result of the combination of all those elements of variation and not only of a given one, and thus one may counteract the effect of another. This may well explain this apparent contradiction.

Besides this, when strains are phylogenetically examined, it can be observed that the same isolates can cluster differently when different viral proteins are looked upon. In our opinion, these results were highly suggestive of differential selective pressures in different genes, potential recombination or reassortment. At this point we are unable to clearly determine the cause. The functional role of the specific nsps, such as nsp1α/β, nsp2, nsp4, nsp7, and nsp11, is the modulation of host immune responses to PRRSV infection and the nsp3–8 region has been identified as containing major virulence factors (Fanga and Snijderb, 2010). In consequence, it is not uncommon observing the highest genetic diversity in those target viral regions suffering direct pressure of the host defence mechanisms. Interestingly, nsp9 seems to represent the whole virus better than

any other else protein. As nsp9 encodes for a virus-encoded RNA-dependent RNA polymerase and it is responsible of the replication of single-stranded RNA viruses with genomes of positive polarity, this protein seems to be the most conserved among strains.

The abovementioned variability most probably will also affect antigenic properties of the virus, such as the induction of NA by GP5 (Plagemann et al., 2002) and recently by GP4 (Vanhee et al., 2010). When the predicted viral proteins were analyzed for the variation in known linear B-epitopes, it could be seen that in the set of linear epitopes reported by Oleksiewicz et al. (2001) only ES2 and ES5 were relatively conserved and ES3 and ES4 can be non existent in field strains. This result suggests that the possibility of constructing a marked differential vaccine by deleting some of the linear epitopes of the nsp2 cluster is at least, extremely difficult. On the other hand, we have made an approach considering linear B-epitopes but to have a full image relating antigenicity with sequence conservation, the tertiary protein structure also matters. It could be possible that tertiary structure-rich regions could interfere with the presentation of strictly linear epitopes to porcine B cells and predominate for instance in functional protein domains. Moreover, it is difficult to predict whether or not amino acid changes would correspond with changes in the recognition by monoclonal antibodies of these linear epitopes. Thus, prior to diagnostic exploitation, more information is needed about the biological significance of the abovementioned changes as well as about the interindividual variability in anti-ES responses.

In summary, taking together, the results of the present study reinforce the notion (Stadejek et al., 2002, 2007, 2008) that genetic diversity in PRRSV is very high and shows that nsp1**,** nsp2 and ORFs 3 and 4 suffer the highest degree of variation within the viral genome. This variability also affects most of the known linear B-epitopes of the virus and may have also an impact on the biological properties of the virus. Also,

the present paper shows that different strains can have substantially different immunobiological properties. These data can contribute to the understanding of the complexity of PRRSV.

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Table 1. Characteristics of PRRSV strains used in the present study.

Strain reference	Country	Year of isolation	Clinical signs (field)	Tissue	Maximum titer in PAM* ($TCID_{50}$/ml)
2982	Spain	2005	Respiratory (weaners)	Lung (weaner)	$10^{4.7}$
3249	Spain	1992	Abortions	Serum (sow)	$10^{4.7}$
3256	Spain	2005	Respiratory (weaners)	Lung (weaner)	$10^{4.7}$
3262	Spain	2005	Respiratory (weaners)	Lung (weaner)	$10^{5.9}$
3266	Germany	1992	Abortions	Serum (sow)	$10^{7.5}$
3267	Portugal	2006	None (boar)	Serum (boar)	$10^{7.3}$

*PAM = Porcine alveolar macrophages

$TCID_{50}$ = Tissue culture infectious dose 50%

Table 2. List of oligonucleotide primers utilized for amplifying the ORFs of PRRSV strains.

Fragment	Primer pair	Primer sequence [a] (5'to 3' sense)	Annealing T[a] (°C)	Primer location [b]
ORF 1	1.0F	CCTGTTCTAGCCCAACAGGT	55	93
	1.0R	AGCCGCGCTCGTAAACT	55	
	1.1F	TGCTGATGTTTTGTACCGTGA	55	524
	1.1R	CAGCACTTGCCATCAAACAC	55	
	1.1B-F[c]	TTCCTCCAACGACTTGTGARGGTTGC	55	501
	1.1B-R[c]	CCATGCACGCCCCACTTGGTTTG	55	
	1.2F	GTGTTTGATGGCAAGTGCTG	55	1032
	1.2R	ATTATGGCGGCAAGGACAT	55	
	1.3F	GACAGACGGGTCTTGTGGTT	55	1493
	1.3R	CATTTTTGCGGAACAACCTC	55	
	1.4F	GAGGTTGTTCCGCAAAAATG	55	2007
	1.4R	CTGGTTGGGACAAATCCAGT	55	
	1.4B-F[d,e]	TGTCCGAGYTCYAAACAGGCCATG	51	2079
	1.4B-R[d,e]	CGGCATCAGAACCTGGGTTGT	51	
	1.5F	ACGAACCACTGGATTTGTCC	55	2518
	1.5R	GAGTTCGGCAAATTCGAAAG	55	
	1.6F	TTCTCCGCACAAGCCTTAAT	55	3021
	1.6R	CTGATAGACCCCGCGAGAC	55	
	1.6B-F[d,e]	CCTGGACCAGCCTTTAGATCT	50	2729
	1.6B-R[d,e]	CTGGGAGATGGGAGACAATTTC	50	
	1.7F	GGGCGGGAGTTGGAAA	55	3488
	1.7R	CGTGACCCACCGAGTAACTT	55	
	1.8F	CGGTGGGTCACGTTATCTCT	55	3951
	1.8R	AGCCACAAAAGTGTCCGAAT	55	
	1.9F	TGGACCAGCCTACACCTGA	55	4378
	1.9R	TGGGTCAGCGGTTCCTC	55	
	1.10F	GACCCATGGTGTTCAAATCC	55	4716
	1.10R	TGTAATAATTCCGGCAACCTG	55	
	1.11F	TCTCAGCCGTGGCACA	55	4819
	1.11R	CGGTGTTAAGGCAGGGTTT	55	
	1.12F	CCTGCCTTAACACCGTGAAT	55	5323
	1.12R	CCAAGTCACTCGGAATGGAT	55	
	1.13F	CCTAGCCTCCGTCCCTGTA	55	5885
	1.13R	TAGGAAGAAGGCGCTTGAAA	55	
	1.13B-F[d]	CGAGCGTTCCCCTTGGAGACAT	55	5883
	1.13B-R[d]	CAGCCGTTAGGGACTCGTTACTCA	55	
	1.14F	TTTCAAGCGCCTTCTTCCTA	55	6385
	1.14R	GCGGACCATTCTCAAAAAGA	55	
	1.14B-F[c,f]	GTGGCTATTCAAATACCGGTGC	45	6332
	1.14B-R[c,f]	TTCATCCTCTCAACTTTGAGATC	45	
	1.15F	CCCCTCTTTTTGAGAATGGT	55	6901
	1.15R	AAAGCCTGTTCAGTGGTCAA	55	
	1.16F	TTGACCACTGAACAGGCTTT	55	7383
	1.16R	GATTGCGGACTTGGTGTCTT	55	
	1.17F	CAAGACACCAAGTCCGCAAT	55	7916
	1.17R	GGTTTTGGGCTTGGAACAGT	55	
	1.17B-F[c]	TGTTAGGGGGGATCCTGAGC	52	7840
	1.17B-R[c]	CCAAGCCTTCTTCATGAATGCCTG	52	
	1.18F	GTTCCAAGCCCAAAACCAG	55	8400
	1.18R	CTCGACCCACCAGTGGTAAT	55	
	1.19F	ATTACCACTGGTGGGTCGAG	55	8964
	1.19R	GGTGACTGACACTGCGAACA	55	
	1.20F	CGCAGTGTCAGTCACCTGTT	55	9480
	1.20R	CACACAAGGGGTGTTTTGG	55	
	1.21F	CCAAAACACCCCTTGTGTGT	55	10047
	1.21R	CGAAATCGAGATGGACCTGT	55	
	1.21B-F[e]	GTGTCGTACCTCGATGAGGC	55	9989
	1.21B-R[e]	GCCCGAGTGATGGCTACAAG	55	
	1.21-22F	CTTGTAGCCATCACTCGGGC	50	10400
	1.21-22R	CCCAGATTATGTGCCACTTGCG	50	
	1.22F	CTCTCTTAGCCGCTTGTTCG	55	10623
	1.22R	GAGTCCTTAGGCAGGGACCTA	55	
	1.23F	CCTACCTAGGTCCCTGCCTAA	55	11107
	1.23R	GATTGCCCAATCCTCAGTGT	55	
	1.24F	CAATCCTTGCACGCCGTAT	55	11604

	1.24R	CAAGTGGGAAACTCGCATGT	55	
	1.24B-F[c]	ACCTGGCAGTGACACCGTATGA	52	11463
	1.24B-R[c]	GAAAGCGTCCACGAACAGCTGGC	52	
ORF2	2F[g]	CTGGCACAGAATTGCAGGTA	55	11724
	2R[g]	GCACACTGATGAGCCATTGT	55	
	2-3F	TGCTCCGCGCTTCTCCGTTCG	50	11951
	2-3R	ACATAGCGTAGAGCTGGAATTCG	50	
ORF3	3F[g]	ACAATGGCTCATCAGTGTGC	55	12401
	3R[g]	TGAAGCCTTTCTCGCTCATT	55	
ORF4	4F[g]	AGCGTGACCATGATGAGTTG	55	12654
	4R[g]	AAAAGCCACCAGAAGCAAGA	55	
	4B-F[d]	GACGGGGGYAATTGGTTYCA	47	12920
	4B-R[d]	CGCCCCAATTTSTGAGAACATCTC	47	
ORF5	L1F[g]	TGAGGTGGGCTACAACCATT	55	13445
	L1R[g]	AGGCTAGCACGAGCTTTTGT	55	
ORF6	6F[g]	GTCCTCGAAGGGGTTAAAGC	55	14037
	6R[g]	CTGTCCTCCCCTAGGTTGCT	55	
ORF7	7F[g]	GGCAAACGAGCTGTTAAACG	55	14555
	7R[g]	AATTTCGGTCACATGGTTCC	55	

[a] Primers were initially designed based on sequence information available for LV (genBank accession number M96262); [b] Numbers correspond to position within the LV genome; T[a], temperature; L, left or forward primer; R, right or reverse primer; [c] primers used for amplifying 3256 strain; [d] primers used for 3262 strain; [e] primers used for 2982 strain; [f] primers used for 3249 strain [g] Díaz et al. 2006 (Virology 351: 249–259).

Table 3. Similarity of the examined ORFs and of the predicted amino acid products of the 6 field strains and 9 European type PRRSV from the GenBank.

ORF/Protein	Length (aa)	Similarity gen. I* (mean± sd)	Range	Substitutions (nº aa; percentage¶)	Similarity gen. II** (mean± sd)	Range
ORF1a	*NA*	*89.5%±4.4%*	*99.8-82.3%*	*ND*	*52.2%±0.4%*	*52.9-51.3%*
Nsp1	385 aa	88.4%±4.5%	100-82.0%	132; 34.3%	52.2%±0.9%	54.3-50.5%
Nsp2	861, 844, 841, 836, 787	83.3%±6.7%	99.7-70.6%	414; ≥48.1%	26.3%±0.8%	28.0-24.4%
Nsp3	447	95.2%±2.2%	100-89.5%	77; 17.2%	56.7%±0.6%	58.0-55.3%
Nsp4	203	95.0%±4.3%	100-90.1%	39; 19.2%	60.5%±0.8%	62.2-58.8%
Nsp5	170	95.5%±2.3%	100-91.1%	28; 16.4%	71.0%±1.7%	73.5-66.4%
Nsp6	16	95.3%±4.4%	100-81.2%	3; 18.8%	78.3%±3.1%	81.2-75.0%
Nsp7	270	96.3%±1.5%	100-92.9%	39; 14.4%	44.6%±0.8%	46.5-43.2%
Nsp8	44	97.5%±2.5%	100-90.9%	5; 11.4%	67.1%±2.0%	70.4-61.3%
ORF1b	*NA*	*93.2%±3.1%*	*99.8-87.3%*	*ND*	*62.7%±0.3%*	*63.3-62.0%*
Nsp9	645	97.9%±1.0%	100-96.1%	48; 7.4%	74.0%±0.4%	75.0-73.2%
Nsp10	442	96.0%±2.2%	100-90.4%	77; 17.4%	63.4%±0.5%	64.7-62.2%
Nsp11	224	97.4%±1.2%	100-94.6%	26; 11.6%	74.7%±1.0%	77.4-72.1%
Nsp12	152	95.0%±2.9%	100-88.1%	26; 17.1%	39.3%±0.6%	40.7-38.2%
ORF2a	*NA*	*93.2%±3.0%*	*99.4-86.9%*	*ND*	*63.8%±0.7%*	*66.9-62.6%*
GP2a	249	93.3%±3.0%	98.7-87.5%	61; 24.4%	60.9%±1.1%	65.2-57.8%
P2b	70	96.4%±3.3%	100-88.5%	9; 12.8%	69.5%±2.0%	75.3-64.3%
ORF3	*NA*	89.9%±5.2%	100-80.5%	*ND*	62.1%±0.7%	63.7-60.6%
GP3	265, 263, 257	87.5%±6.3%	100-77.3%	63; ≥23.7%	54.9%±1.2%	57.3-54.2%
ORF4	*NA*	89.9%±5.0%	100-80.9%	*ND*	64.5%±1.2%	66.8-77.0%
GP4	183, 181, 175	88.5%±5.7%	100-77.0%	57; ≥31.1%	66.8%±2.3%	70.4-59.5%
ORF5	*NA*	91.1%±3.7%	99.8-84.0%	*ND*	60.7%±0.7%	62.4-58.5%
GP5	202	90.0%±3.5%	99.5-84.0%	62; 30.7%	54.7%±1.3%	56.5-50.7%
ORF6	*NA*	*93.8%±3.3%*	*100-88.1%*	*ND*	*69.2%±0.7%*	*69.2-71.2%*
M	173	94.6%±2.8%	100-89.0%	32; 18.5%	78.8%±1.2%	81.6-75.2%
ORF7	*NA*	*94.7%±2.4%*	*100-89.4%*	*ND*	*61.7%±0.7%*	*63.0-60.0%*
N	127	95.0%±3.3%	100-88.2%	24; 18.9%	58.6%±1.1%	61.0-55.7%

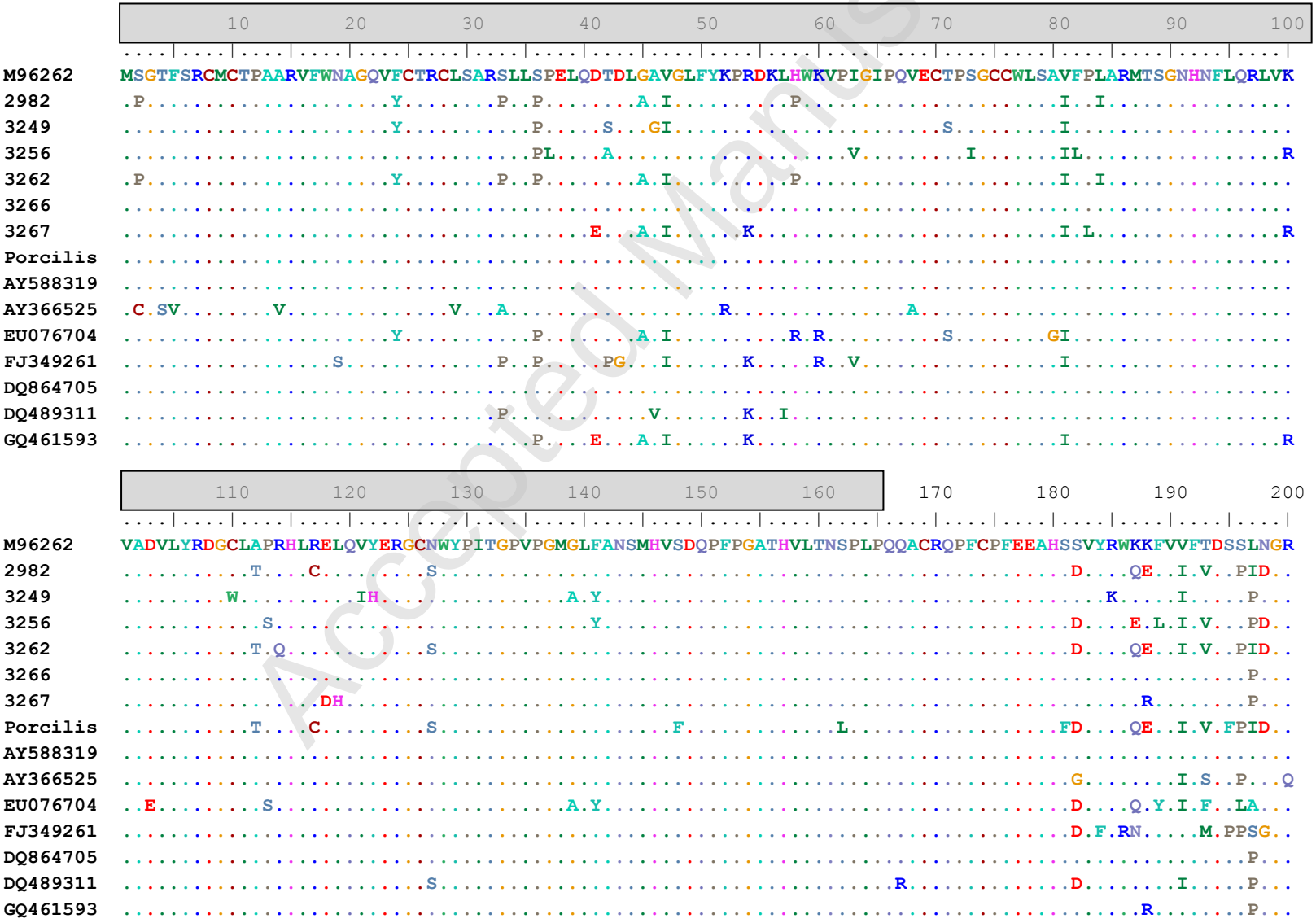
* **Similarity gen. I:** Similarity among genotype I strains; ** **Similarity gen. II:** Similarity of genotype I with genotype II strains; ¶ Percentage was calculated over the longest aa length. NA; not applicable. ND; not done.

Table 4. Cytokine levels in cultures of peripheral blood CD172a+ cells (IFN-α, TNF-α and IL-10) or porcine alveolar macrophages (PAM) (IL-1, IL-6 and IL-8) stimulated with different viable PRRSV isolates at 0.01 multiplicity of infection as determined by capture ELISA. Values are shown in pg/ml.

	Cytokine levels in cell culture supernatants (pg/ml)					
Strain	**IFN-α**	**TNF-α**	**IL-1**	**IL-8**	**IL-6**	**IL-10**
2982	Neg.	376	304	>8000	Neg.	212
3249	Neg.	103	216	>8000	Neg.	Neg.
3256	Neg.	437	1232	>8000	Neg.	723
3262	Neg.	1119	411	>8000	Neg.	461
3266	Neg.	Neg.	301	>8000	Neg.	Neg.
3267	Neg.	Neg.	302	>8000	Neg.	Neg.
Mock cells*	Neg.	Neg.	Neg.	<1000	Neg.	Neg.

*Medium-stimulated cells.

Figure 1. Graphic view of the alignment of the predicted amino acid sequences of non structural protein 1 (1α and 1β) for the examined PRRSv strains. Shadowed upper box shows nsp1α. Linear B-epitopes described by Oleksiewicz *et al.* (2001) are shown as dotted boxes with the label ESx.



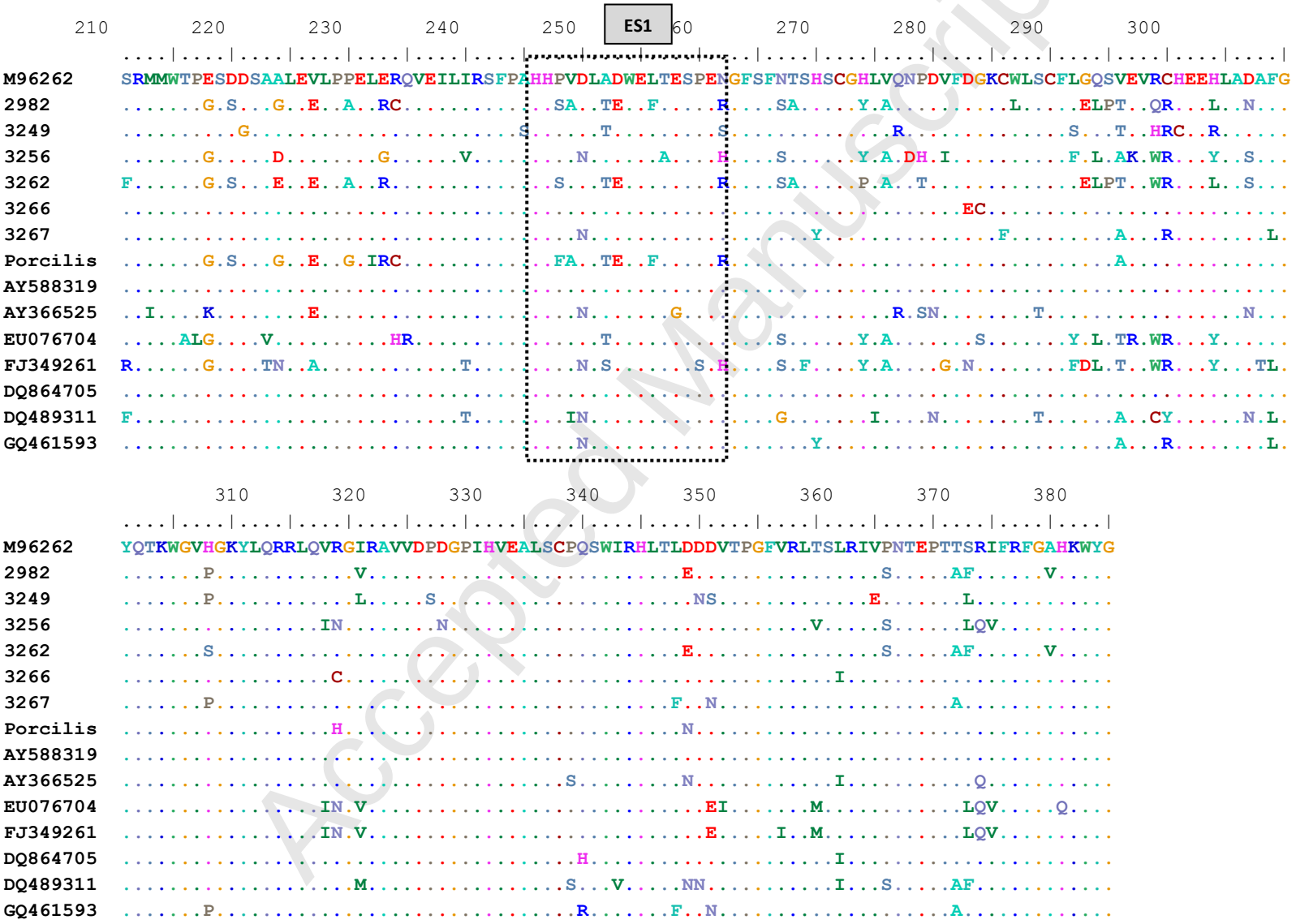
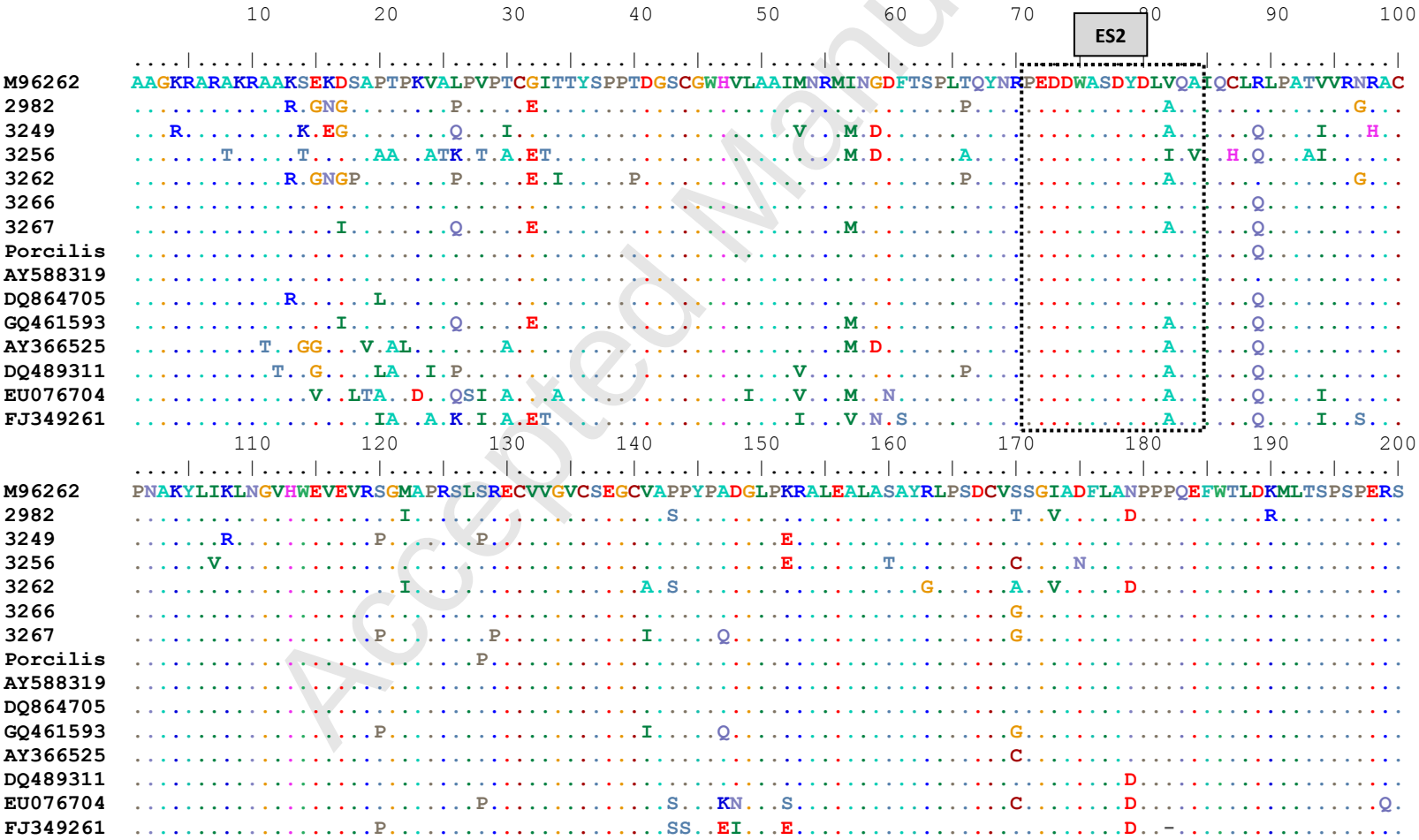
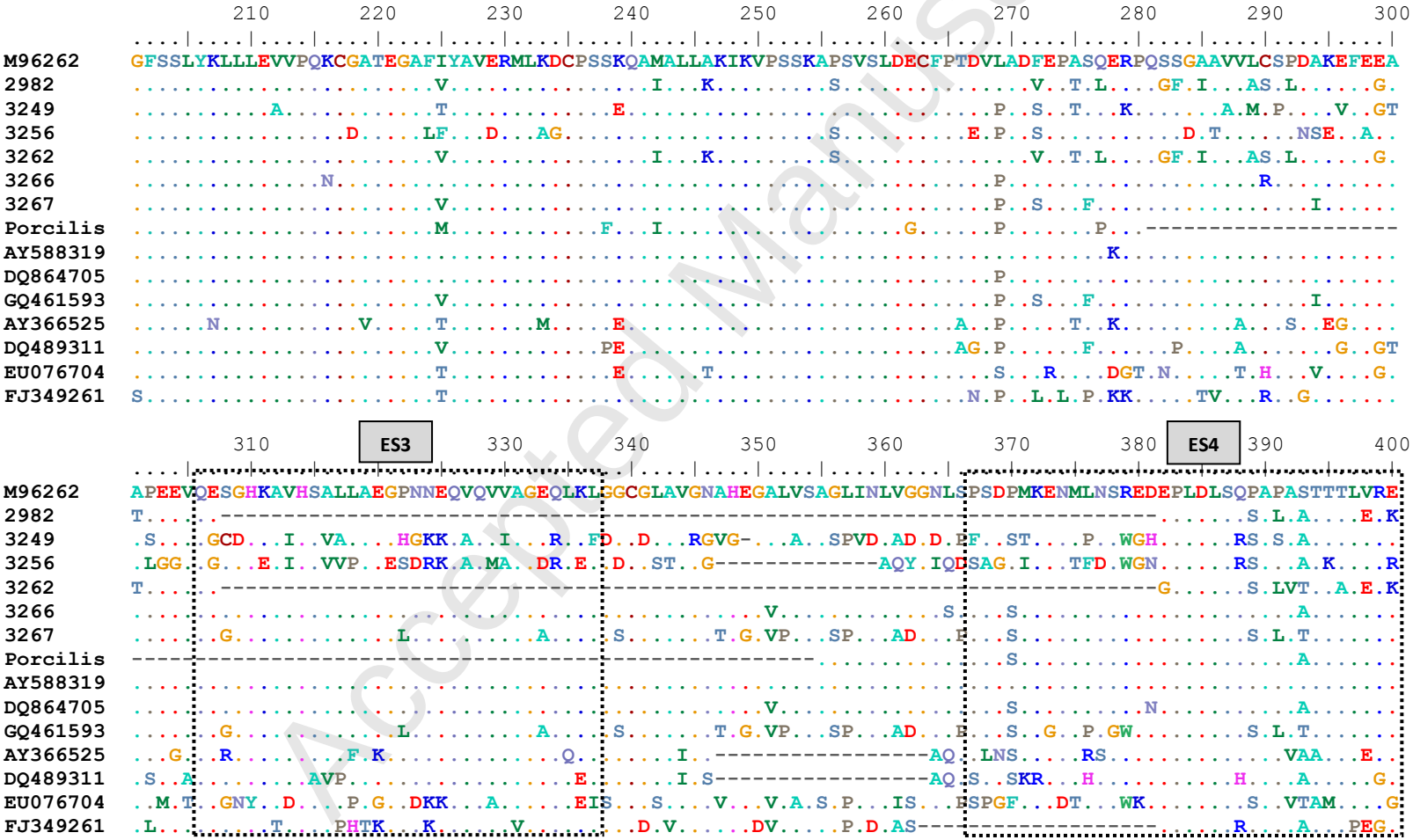
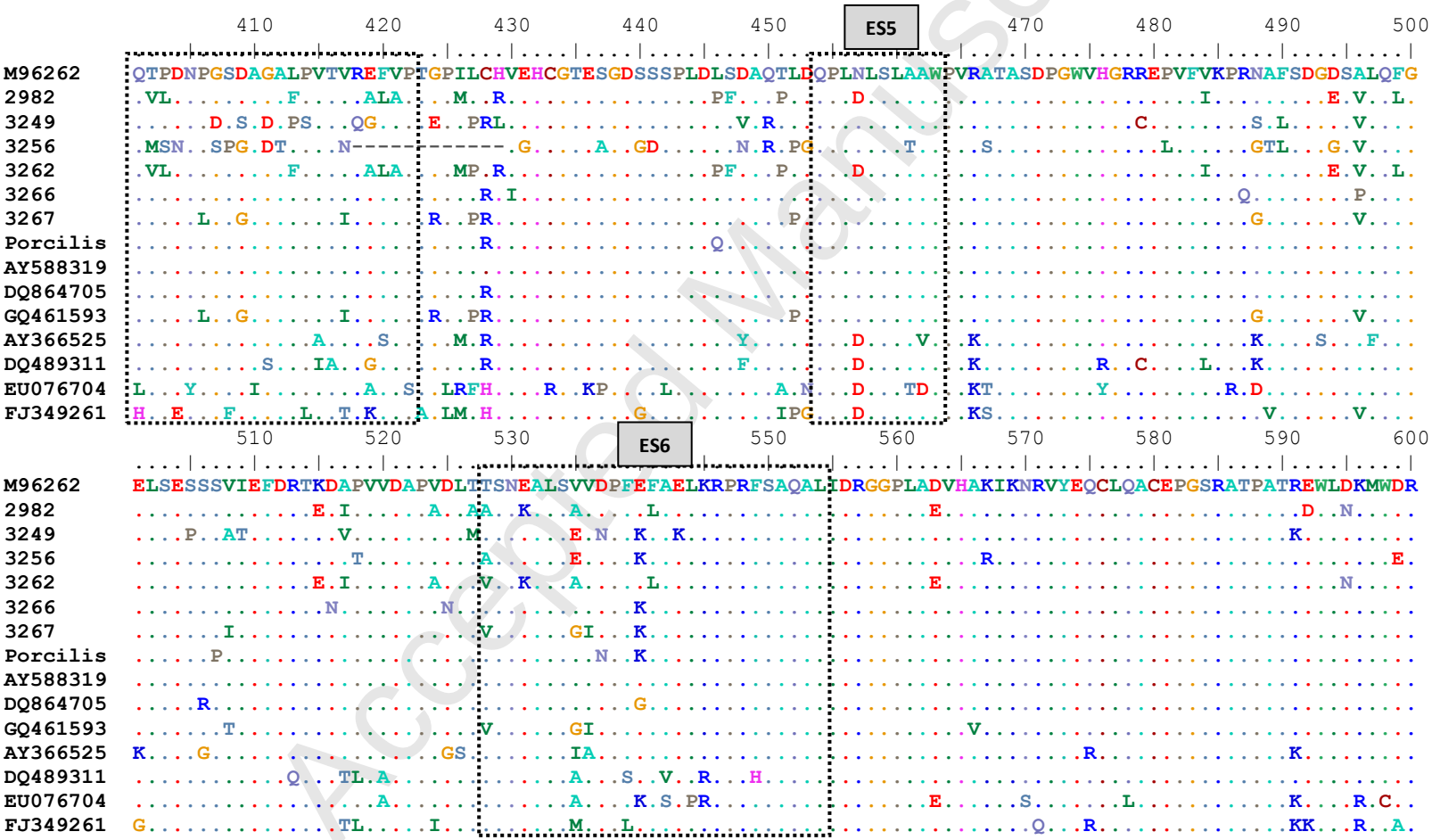
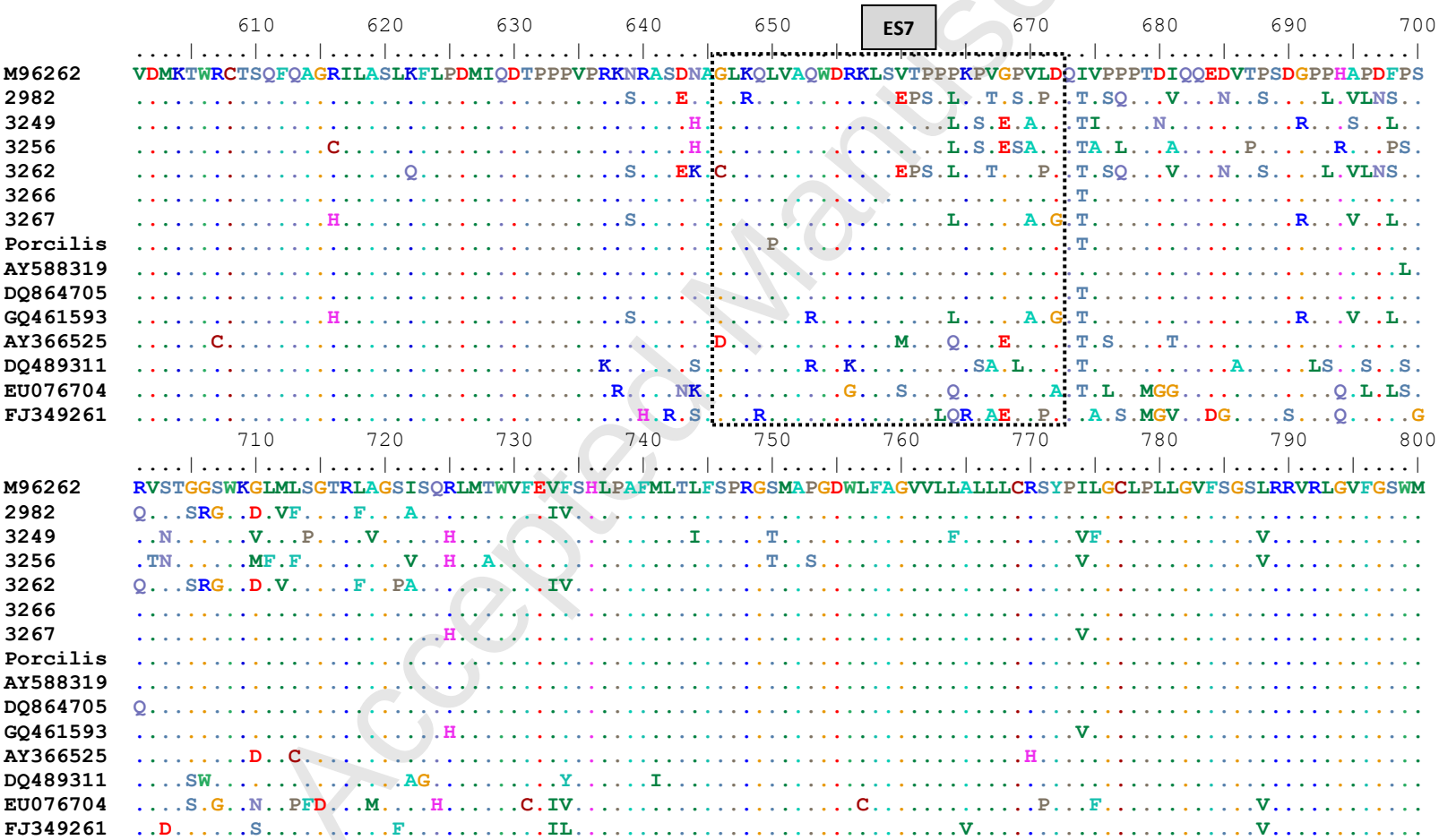


Figure 2. Graphic view of the alignment of the predicted amino acid sequences of non structural protein 2 for the examined PRRSv strains. Linear B-epitopes described by Oleksiewicz *et al.* (2001) are shown as dotted boxes with the label ESx.









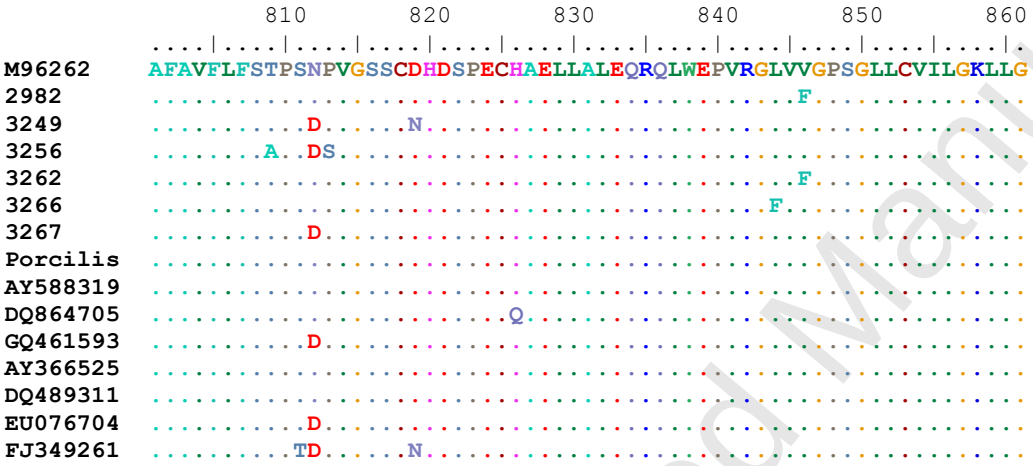


Figure 3. Partial graphic view of the alignment of the predicted amino acid sequences of GP3 of the PRRSV strains examined. The dotted box shows the epitope reported by Oleksiewicz *et al.* (2000).

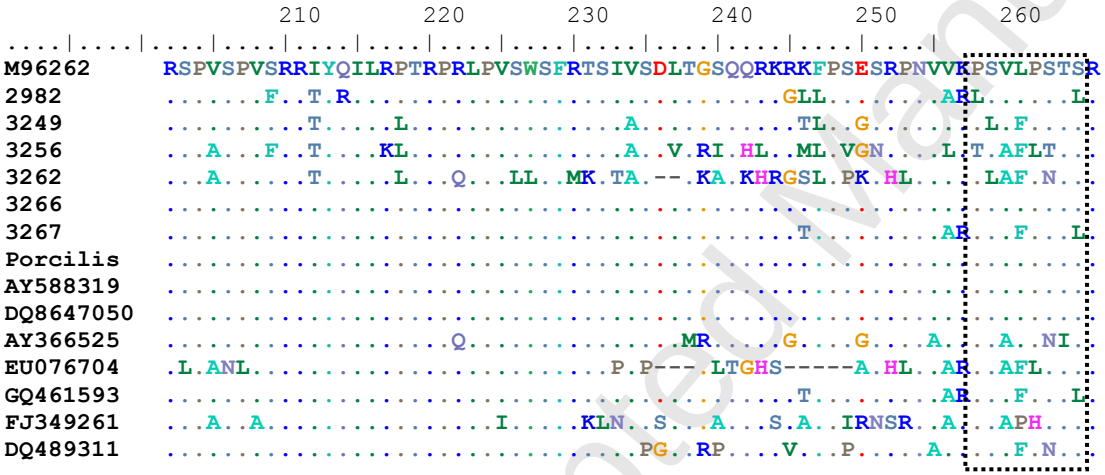


Figure 4. Graphic view of the predicted amino acid sequences of GP4. The dotted box show the location of the neutralization epitope reported by Meulenberg *et al.* (1997).

Figure 5. Topology of the synonymous-non synonymous substitution rate in codons (ORFs1-7) of 15 European type PRRS strains. Annotation in the lower part of the graph indicates topology of each ORF. Boxes located in the center show the location of non structural proteins in ORF1a/1b. Y-axis show the rate of synonymous-non synonymous substitutions per codon; X-axis show the number of codons starting at nsp1.

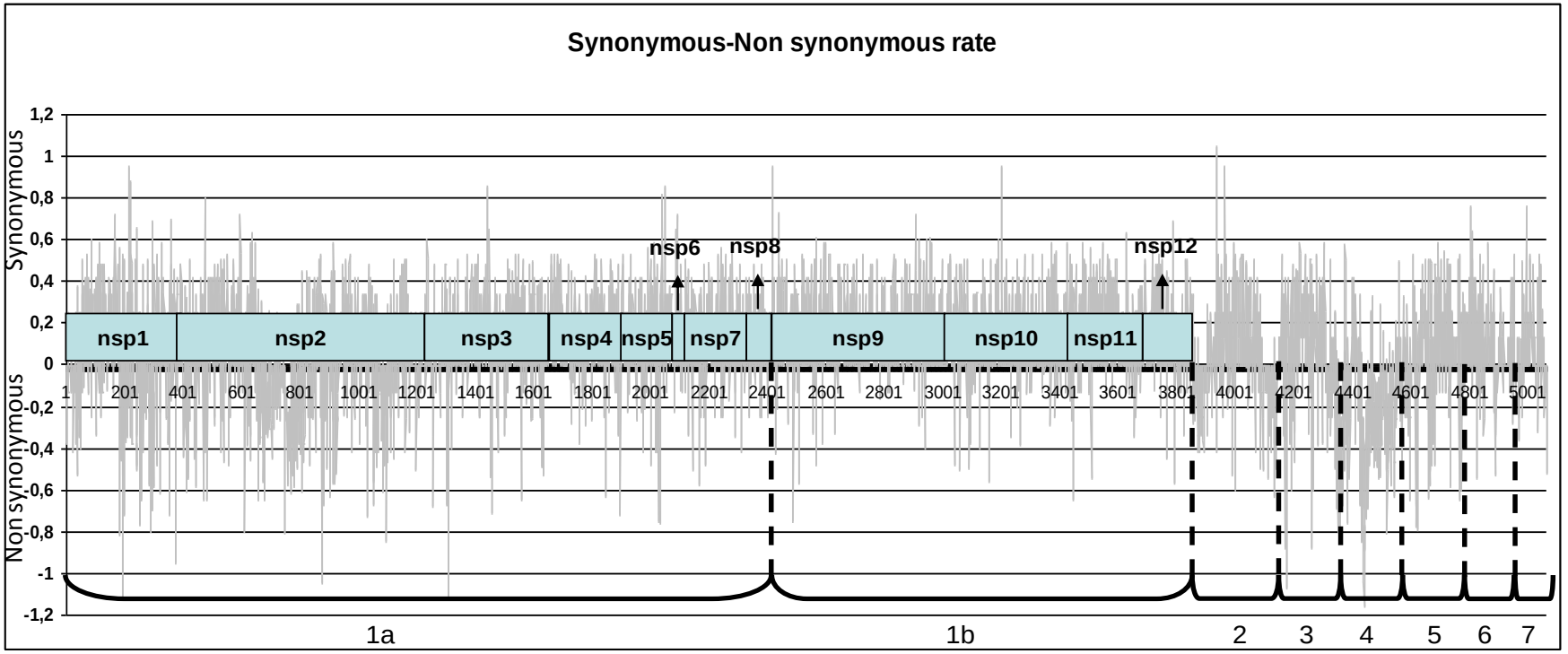


Figure 6. Mid-point rooted tree based on the genomic sequence of nsp9, on full ORFs1-7 sequences and ORF5 and ORF7 PRRSV sequences. Bootstrap values are shown in the branches.