Full title: Bottlenecks in the transmission of *Porcine reproductive and respiratory syndrome virus* (PRRSV1) to naïve pigs and quasi-species variation during infection in partially immune pigs

Short title: PRRSV1 quasi-species transmission and variation

by

Martí Cortey[1]#, Gastón Arocena[1], Emanuela Pileri[1], Gerard Martín-Valls[1], Enric Mateu[1,2]

[1] Departament de Sanitat i d'Anatomia Animals, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Spain

[2] IRTA, Centre de Recerca en Sanitat Animal (CReSA, IRTA-UAB), Campus de la Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Spain

*Corresponding author (e-mail): Martí Cortey (marti.cortey@uab.cat)

Keywords: Porcine reproductive and respiratory syndrome virus, PRRSV, deep sequencing, transmission events, founder variants

Abstract

The existence of bottlenecks during infection of *Porcine reproductive and respiratory syndrome virus* (PRRSV) was studied in an experimental one-to-one model of transmission in pigs. Besides, the differences between viral quasi-species in vaccinated pigs that developed shorter or longer viremias after natural challenge were analysed. The results consistently reported the existence of bottlenecks during transmission. Several positions along the PRRSV genome were identified as being selected in partially immune animals that developed short viremias. Those positions accumulated in GP2, nsp9 and M proteins and resulted in changes in the protein structure and in the interactions of those proteins with their targets. The fact that the affected proteins are known targets of the immunity against PRRSV suggested that the immune response selected those changes. This pig model can be useful for the study of other pathogens of interest in animals and humans.

35 **Author summary**

36 Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically
37 important disease of pigs. It is caused by PRRS virus (PRRSV), a positive-sense, single-stranded
38 RNA virus in the *Arteriviridae* family within the order *Nidovirales*. Here, we study the existence
39 of bottlenecks during disease transmission and the differences between viral quasi-species in
40 vaccinated pigs that developed shorter or longer viremias after natural challenge. Our results
41 consistently report the existence of bottlenecks during PRRSV1 transmission and identify several
42 mutations along the viral genome selected by the host immune response that can be clear
43 targets for new vaccine development.

Introduction

The comprehension of how pathogen transmission occurs is key to understand infectious diseases. Most often, the route of transmission, the portal of entry and the minimum infective dose – when known –, are common features to characterize transmission. However, it is increasingly evident that transmission is an extremely complex phenomenon. For example, because of the existence of bottlenecks during host-to-host transmission [1]. A bottleneck can be defined as a sharp reduction in the size (population bottleneck) or the diversity (genetic bottleneck) in the pathogen population effectively transmitted. The existence of such bottlenecks must be examined considering the portal of entry in the recipient host and the source of the pathogen (blood, nasal secretion, faeces, etc.). Since a pathogen may be present in different tissues, organs, or fluids, each one might be considered a compartment with its own particularities. The pathogen population contained in the compartments where the transmission to the next host occur is termed transmissible population; whereas the successful settlers in the recipient host are called founder variants or transmission founders. The location, size and genetic diversity of the transmissible population can influence the founder population after a transmission event [2-4].

Successful transmission founders can be though as either the result of a non-selective bottleneck – namely, the lucky few that crossed by chance the portal of entry –, or viewed as a selective bottleneck, where only the variants fit enough to cross the portal of entry are transmitted. In both cases, pathogen genetic diversity in the very early phases of the infection would be limited, but with different biological significances. In the case of RNA viruses – that exist as quasi-species – those different scenarios could imply very different outcomes. While the first case – a non-directional unspecific bottleneck – would produce a new quasi-species cloud from randomly selected variants; a directional bottleneck would promote the expansion in the recipient host of variants arising from founders already fit for transmission, although not necessarily the fittest, neither the most efficient for replication in the host.

There are other of factors, such as the immune status of the host that may influence the diversity of a quasi-species. For example, in *Hepatitis C virus* (HCV), continuous diversification has been considered the mean by which the virus escapes the immune system and establishes persistent chronic infection [5]. However, this is an extremely complex phenomenon, where other factors, such as antigenic cooperation between intra-host variants, may permit immune adaptation leading to the co-existence of viral variants with different bind capacities to antibodies, or to be attacked by the cell-mediated immunity [6, 7].

The *ex vivo* study of founder variants and the quasi-species evolution in humans is challenged by the difficulty of determining the precise timing of transmission and the associated quasi-species distribution in the donor. However, the existence of systemic animal diseases caused by RNA viruses with high substitution rates creates an opportunity for examining, in a more controlled environment, transmission bottlenecks and quasi-species variation.

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically important disease of pigs. It is caused by PRRS virus (PRRSV), a positive-sense, single-stranded RNA virus in the *Arteriviridae* family within the order *Nidovirales*, exhibiting one of the highest substitution rates observed [8, 9]. Experimental models to study PRRSV are well known and have been applied to study transmission [10, 11]. Interestingly, the immune response against PRRSV is unusual, since neutralizing antibodies appear late and cell-mediated immunity has an erratic course for weeks. However, after several weeks of viremia, the virus is confined to the lymphoid tissue and eventually cleared [12, 13]. Pre-formed neutralizing antibodies may protect against the homologous infection in a dose dependent way [14], although that heterologous protection cannot be foreseen [15]. Besides, there is a large individual variation in the immune response [16]. As a result, when a vaccinated animal is challenged, viremia usually develops, but generally of lesser duration than in a naïve animal.

In the present study we used Next Generation Sequencing (NGS) to analyse the quasi-species diversity and evolution in a transmission model of PRRSV in order to: i) characterise and compare the transmissible population and the founder variants in intra-nasally inoculated and naturally infected by contact animals, ii) compare the diversity at early and late phases of viremia and, iii) identify the differences in the viral quasi-species between pigs with partial immunity developing short and long viremias after being in contact with infected pigs.

Results

The RNA NGS method was suitable for assessing viral quasi-species

Deep sequencing results for PRRSV1 from sera with high viral load and cell culture supernatants of singly passaged serum samples produced similar viral quasi-species. The estimated error rate between sera and isolated virus ranged between 1 and 3 nucleotides for every 10,000 nucleotides inferred. This rate was considered an acceptable bias. The quality scores (QC) of the NGS runs were above 30 in all the analysed samples, yielding a depth of reads for viral sequences above 115 in all cases. With this depth, variations in the range of percentage units could be determined.

The characterization of PRRSV transmission events supports the existence of bottlenecks

The transmission experiment scheme and results are summarized in Figure 1. In 8/9 of the intra-nasally inoculated naïve pigs, the viral population in blood at the onset of viremia showed lower diversity compared to the initial inoculum. Similarly, in 4/5 of the naïve pigs infected by directed contact with a seeder, the observed nucleotide diversity was lower compared with their seeder counterparts. As a whole, and assuming the limitations of the method, the results pointed to a reduction in the diversity of the founders compared to the transmissible population, supporting the existence of a bottleneck during PRRSV transmission events.

Taking advantage of the availability of serial samples during the virological course of each animal, it was possible to compare the diversity at different stages (Table 1). After the initial

119 reduction during transmission, nucleotide diversity increased in most pigs analysed, although in

120 some animals this was not the observed pattern.

121 **Table 1.** Variation of the nucleotide diversity (π) between days of viremia. The table shows the
122 difference in nucleotide diversity between samples of the same animals in different days of the
123 virological course. The difference of nucleotide diversities was calculated by subtracting the π
124 value of a given day from the π value the previous examined day. For the first day of each animal
125 (in red) the difference was calculated with regards to the inoculum (inoculated animals) or with
126 regards to the diversity of the donor on the likely day of transmission (transmission by contact)

Group	Animal nº	Viremic day	π	Difference between consecutive samples
Inoculum produced in PAM	N.A.		0.0127	
Inoculated animals	1	1	0.0045	-0.0082
		6	0.0182	0.0137
		13	0.0239	0.0057
	2	1	0.0091	-0.0036
		6	0.0124	0.0033
		13	0.0115	-0.0009
	3	1	0.0064	-0.0063
		13	0.0075	0.0011
	4	6	0.0117	-0.001
		13	0.0528	0.0411
	5	1	0.0123	-0.0004
		13	0.0132	0.0009
	6	1	0.0305	0.0178
		6	0.0308	0.0003
		15	0.0097	-0.0211
	7	1	0.0072	-0.0055
		13	0.0524	0.0452
	8	1	0.0064	-0.0063
		06	0.0152	0.0088
		13	0.0088	-0.0064
	9	1	0.0091	-0.0036
		6	0.0041	-0.005
		13	0.0335	0.0294
Naïve infected by contact	1	1	0.0031	-0.0151
		6	0.0119	0.0088
		22	0.0178	0.0059
	2	1	0.0108	-0.009
		8	0.0091	-0.0017
		15	0.0109	0.0018
	3	3	0.0134	0.007
		10	0.0165	-0.0031
	4	1	0.0092	-0.0025
		5	0.0065	0.0027
	5	3	0.0069	-0.0054
		10	0.0099	0.003

127

128 *Preferred and un-preferred transmitted variants are differentially distributed along the PRRSV*

129 *genome*

Mean frequency differences for each nucleotide in every genome position were calculated for the transmission events where naïve acceptors were infected by seeder pigs (Figure 1B). For this section we used only data from pigs infected by contact, since for the inoculated pigs (Figure 1A), the Porcine Alveolar Macrophage (PAM) propagated inoculum might have shown some level of adaptation to the cell passages and this might have masked the true changes associated with transmission fitness. Figure 2 shows the mean increase or decrease in the frequencies of each nucleotide per position (only values larger than 5% are shown), between the quasi-species of the transmissible population and the transmitted founders. An increase in the nucleotide frequency was associated with a preferentially transmitted variant, while a decrease was identified as an un-preferred transmitted one. Along the PRRSV1 genome, 65 variants in 32 positions were detected (32 preferred and 33 un-preferred). Nineteen nucleotide variants produced synonymous changes, while 13 variants induced potential amino acid changes.

A salient feature of the picture was the non-random distribution of the affected positions: 10 in nsp2 region (31%, 4 synonymous and 6 non-synonymous), 3 positions in nsp4 (9%, 2 synonymous and 1 non-synonymous), 3 in nsp9 (9%, all synonymous), 6 in nsp10 (19%, 3 synonymous and 3 non-synonymous) and 3 in ORF5 (9%, 1 synonymous and 2 non-synonymous).

Next, we examined and related the heteronymous changes with the regions and known features of the encoded proteins. The six amino acid changes detected within nsp2 fall in the two hypervariable regions flanking the papain-like cysteine protease domain, with one change (Gln-336-Lys) located in the B-Cell epitope site 3 proposed by [17] and a second one (Tyr-736-His) falling in a non-conserved epitope inducing IFN-γ and IL-10 responses, as reported by [18]. In nsp4, an Ile-142-Leu change is located in the middle β-barrel domain II of the main PRRSV proteinase 3C-like protease, but the change did not result in any substantial change in the 3D structure of the protein. The single change detected in nsp5 – Leu-32-Phe – was located in a transmembrane domain. Regarding nsp10, 2 out of 3 amino acid changes identified in this protein fall in the Zinc-binding domain, while the third was located in the C-terminal domain,

156 downstream the helicase. One of the affected residues, located at position 46, preferentially
157 changed from Ser to Gly during the transmission events. Finally, in GP5 two nucleotide
158 mutations in the first and second position of a codon leaded to the change Ile-36-Asp in the
159 hypervariable ectodomain of GP5, just after a signal peptide cleavage site. None of the
160 abovementioned changes produced a relevant structural change in any protein.

161 *Differences in viral quasi-species between short- vs long-viremia pigs are mostly located in nsp9*
162 *and ORF2*

163 In PRRS, immunity against heterologous viral strains is considered partial and, in consequence,
164 the infection of vaccinated animals is possible if a heterologous strain is used. After examining
165 transmission to naïve pigs, we compared the viral quasi-species in two groups of partially
166 immune pigs infected by contact from seeder penmates in a one-to-one basis. Those partially
167 immune animals developed viremias that were classified as long (*LV*) or short (*SV*). The results
168 indicated a different distribution of the changes in nucleotide frequencies between groups along
169 the viral genome (Figure 3). Forty-five of the 55 positions (81.8%) identified were located in the
170 nsp9 (37 positions, 64%) and ORF2 (8 positions, 15%); while these two proteins only account for
171 17.8% of the nucleotide positions in the viral genome. Remarkably, in all these 55 positions, the
172 nucleotide variants characterising the *LV* group coincided with the nucleotide present in the
173 original strain causing the infection, while the *SV* group always showed a different nucleotide.
174 When the nucleotide variants present in the *SV* and *LV* were translated, most variants (43)
175 generated the same amino acid (blue dots in Figure 3), but 12 introduced amino acid changes: 2
176 located in nsp2, 5 in nsp9, 3 in ORF2 and 2 in ORF6 (red dots in Figure 3).

177 Again, non-synonymous changes were examined for potential biological significance. The two
178 changes identified in nsp2, Ala-392-Thr and Pro-669-Ser, fall in the B-cell epitopes 4 and 7
179 described by [17]. In nsp9, one of the key enzymes for PRRSV synthesis, all the amino acid
180 changes identified were located in the viral RNA-dependent RNA polymerase (RdRp), encoded

by the C-terminal domain. The comparison of the RdRp 3D structures between *SV* and *LV* groups (inferred with SWISS-MODEL), indicated that the amino acid changes Lys-338-Arg and Lys-641-Arg do not produce any evident structural change. On the contrary, the change Gly-387-His, caused by nucleotide changes in the three positions of the codon, induced a structural change. The presence of a 387Gly in *SV* group resulted in the formation of an α-helix between residues 384-388 and reduced the positive charge at the opposite side of the active centre of the protein where Mg^{2+} molecules bind. (Figure 4).

In GP2, the three amino acid changes identified, namely Gln-146-Arg, Val-151-Ala and His-184-Arg. fall in the ectodomain, slightly downstream the linear B-cell epitopes proposed by [19] and [20]. The amino acid changes reported for this protein implied more positively charged residues in *LV* compared to *SV* group.

Finally, the last two amino acid changes, Asp-10-Asn and Stop-25-Tyr, are located in the short stretch exposed at the virion surface in the N-terminal ectodomain of protein M, encoded by ORF6. The Asp-10-Asn imply a charge modification from neutral (*LV*) to negative (*SV*).

Discussion

With the introduction of NGS technologies, the experimental analysis of viral genetic diversity has changed dramatically. Due to its massively parallel approach, NGS generates millions of reads that cover every nucleotide position from thousands to hundreds of thousands times. Hence, low-frequency variants within viral quasi-species can be adequately detected and characterized [21] and it is possible to see variations that, although significant, would remain unnoticed using Sanger sequencing. However, the usefulness of NGS for viral diversity estimations depends crucially on the quality of the sample and on the procedure to prepare it [22]. In the present work we applied a tailor-made NGS method to characterize the diversity of PRRSV quasi-species. The method omitted the PCR amplification step and used instead a singly passage in PAMs when needed because of the low amount of virus present in many samples. By

206 using PAMs, the same cells that support viral replication in the host, a low bias was generated
207 in a single passage and the error rate produced was also very low. This approach could be useful
208 for other viruses that cannot be analysed directly by NGS from biological samples because of the
209 low titres present.

210 As stated, virus populations may face bottlenecks during the infection cycle [1]. When
211 transmission takes place through a mucosal portal of entry, infection is usually initiated by a
212 limited number of viral particles compared to the total number of particles reaching that portal
213 [4]. Besides population bottlenecks, there are many examples of extreme genetic bottlenecks in
214 RNA viruses such as *Human Immunodeficiency Virus* (see reviews by [4] and [23]), HCV [24-26]
215 and *Simian immunodeficiency virus* [27].

216 In most of the experimentally inoculated pigs and the animals infected in a quasi-natural way by
217 contact with infected seeder pigs, viral diversity was reduced during transmission, supporting
218 the existence of a bottleneck during PRRSV infection. The nature of such a bottleneck is more
219 difficult to precise. The changes in the nucleotide diversity were not scattered randomly across
220 the viral genome, but focused in a few targets. Beforehand, one could think that the structural
221 proteins interacting with the target cells would be the most affected ones, since they are the
222 first interacting with the mucosa surface. Interestingly, this was the case with GP5, the viral
223 glycoprotein establishing the first interaction with porcine sialoadhesin, one of the viral
224 receptors on the macrophage surface [28]. The preferentially transmitted variant introduced a
225 change in position 36 favouring an Asp, a more acidic amino acid. It is difficult to interpret this
226 result but it is located close to a potential glycosylation site and adjacent to the neutralization
227 epitope in GP5 [29]. It is tempting to hypothesize a potential increased interaction between GP5
228 and siloadhesin favoured by the higher polarity of the 36Asp variant. Besides, it is worth noting
229 that all but one of the other favoured changes affected non-structural proteins, pointing
230 towards the selection of variants with different replication characteristics, although the result
231 of the precise amino acid changes could not be ascertained from the literature.

After the initial diversity reduction during the transmission event, the viral diversity of the circulating quasi-species increased in most cases, as expected in an initial expansion of a viral population in a naïve animal. Afterwards, in later stages, diversity could increase or fall but since a detailed characterisation of the immune response at each time point was not performed, the causes are not clear.

The third objective of this work was to analyse the quasi-species differences between two groups of partially immune pigs developing short and long viremia. In the present work, the duration of the viremia was correlated with the titers of neutralizing antibodies against the virus [10]. Therefore, beforehand, main changes were expected to be located in potential targets for the antibodies. Neutralizing antibodies for PRRSV have been reported to be induced by GP2, GP3, GP4, GP5 and M proteins, although immune-dominant epitopes are mainly located in N and nsp2 [14, 30]. In the present case most of the changes in *SV* occurred in nsp9, followed by ORF2 that encodes GP2 (Figure 3). The 3D modelling of nsp9 (RdRp, Figure 4) showed that the introduction of a Gly-387-His after changes in the three nucleotides of the codon, produced a change in the folding of the protein, from linear to α-helix. This change resulted in the absence of a positively charged group opposite to the site of union of Mg^{2+}, and would probably cause a modification in the electrostatic forces involved in the interaction with the NTP channel. It is reasonable to think that such a change would affect the efficiency of RNA synthesis.

Regarding the changes in the ORF2, the variants found in *SV* also resulted in a less charged protein. GP2 is known to interact together with GP3 and GP4 with CD163 [31], the essential cell receptor for PRRSV [32, 33]. Again, this could induce a weaker interaction with the receptor CD163.

Apart from the abovementioned changes in nsp9 and ORF2, an additional interesting change was observed in the M protein (encoded by ORF6). The negative charge present in Asp10 (*SV*) may interfere in the disulphide link between the residue 9 of M protein and the residue 48 of

GP5 [34]. Therefore, potential weaker interactions in the variants present in *SV* pigs with heparan-sulphate, another receptor protein for PRRSV may be induced [35].

In the frame of animals with higher titers of neutralizing antibodies, as observed in the *SV* group [10], the changes reported in GP2 and M could be understood as escape mutations. The lower antibody titers in the *LV* group probably prevented those changes from being positively selected, and the major variants present in the initial inoculum remained the commonest. Similarly, it was proposed that T-cell responses contributed to partial levels of cross-protection in PRRSV, and therefore, the changes observed in nsp9 could be seen as escape variants [36]. Potential T-cell epitopes for PRRSV have been proposed for nsp9 [37], as well as for the RdRp of other *Nidovirales* [38, 39]. The striking fact relates with the fact that those changes in nsp9, GP2 and M would probably result in less efficient viral variants for, either interacting with the cell receptor, or replicating. This scenario suggests that in animals with higher levels of immunity, the variants escaping the immune system are not fit enough to maintain a viable quasi-species cloud, and consequently, the viral population collapses. Accordingly, nsp9, GP2 and M would be clear targets for new vaccine development.

In summary, the present report shows a feasible approach to study transmission events and changes in viral quasi-species during the course of an infection in pigs. The results were compatible with the existence of a transmission bottleneck for PRRSV and showed some targets for understanding the effects of the immune response on viral diversity. This pig model could be used to study human diseases such as influenza and to gain understanding of how transmission of RNA virus occur.

Materials and Methods

Animal experiment

Samples used in the present study were obtained in the course of a previous experiment aimed to determine the transmission of PRSRV in a one-to-one basis [10]. Figure 1 summarizes the first

282 experiment. Two different scenarios were examined in the first experiment regarding
283 transmission. The first one studied animals experimentally infected by the intranasal route with
284 a PRRSV inoculum produced in PAMs (n=9); the second scenario consisted in cases of
285 transmission by contact to naïve pigs (n=5). Those PAMs were obtained from bronco-alveolar
286 washes of lungs collected from four-week old high health pigs. In all cases, samples used were
287 sera collected in the first observed day of viremia for the recipient, usually day 2 after inoculation
288 or contact and when transmission occurred by contact, the closest day of viremia to that event
289 for the donor (usually 1-3 days before the onset of the viremia in the recipient). Additionally,
290 since samples at later viremia stages were available we examined the changes in the diversity
291 throughout the viremic period.

292 In a second experiment, the changes in the viral quasi-species present in blood of partially
293 immune animals (previously vaccinated), that got the infection by contact with seeder pigs was
294 examined (n=11). For this purpose, the last day of viremia was analysed. In this later experiment,
295 animals were seen to develop long viremias (≥7 days), or short viremias (<7 days) and
296 accordingly, were classified in two groups: short viremia (*SV*, n=7) or long viremia (*LV*, n=4). Since
297 in some cases the amount of virus in blood was not enough to proceed directly to NGS, all
298 samples were subjected to a single passage in PAMs in order to maintain similar conditions for
299 all.

300 For the first experiment, the inoculum used was a sixth passage of strain CReSA3267 (Accession
301 Number JF276435). In both experiments, viral load in blood was determined by one step RT-PCR
302 (qRT-PCR) targeting PRRSV ORF7 using the method described by [40].

303 *NGS protocol*

304 Cell culture supernatants were firstly centrifuged up to 14,000 g in order to remove potential
305 debris. Afterwards, total RNA was extracted using the Trizol LS© reagent according to the
306 manufacturer directions. Extracted RNA was assessed by spectrophotometry at 260 nm and 280

307 nm and used in the PRRSV-specific RT-PCR as above for determining concentration of RNA and
308 purity.

309 The assessment of PRRSV diversity within each serum sample was characterised directly from
310 RNA without any previous amplification step using a NGS approach developed by our group. The
311 procedures included: i) the construction of a genomic library for Illumina NGS sequencing using
312 a commercial protocol and reagents (Protocol for use with Purified mRNA or rRNA Depleted RNA
313 and NEBNext® Ultra™ II RNA Library Prep Kit for Illumina®, New England Biolabs), ii) trimming
314 of low quality reads (QC>20 as determined by FastQC©software, Babraham informatics) using
315 Trimmomatic© [41], iii) mapping of the reads against strain CreSA3267 using the Burrows-
316 Wheeler Aligner applying the BWA-MEM algorithm for long reads [42], iv) variant calling with
317 SnpSift© to determine the frequency of each nucleotide at each position of the reference
318 genome and, v) construction of the viral quasi-species in fasta format.

319 *Validation of the procedure, estimation of the PAM passage error rate and quality control check*

320 Given that the samples used were cell culture supernatants, it was necessary to validate the
321 technique for ascertaining the potential bias introduced by the single passage in PAM. For this
322 purpose, four samples with an estimated viral load of >10E6 viral genomes/ml (after
323 quantification by RT-PCR) were directly used in the abovementioned protocol. In parallel the
324 same samples were singly passaged in PAM and the cell culture supernatants were examined
325 similarly. To be subjected to further analysis, a sample should produce a complete genome with
326 at least a depth of 100 reads per position.

327 *Nucleotide diversity estimations and frequency changes per position in the transmission events*

328 For assessing the change in the diversity of viral populations in the different transmission cases,
329 the calculation of the nucleotide diversity (π) was performed using DNAsp [43]. These
330 calculations were comparing respectively: a) the diversity in the PAM inoculum versus the
331 diversity in the samples collected the first day of viremia in experimentally inoculated animals

and, b) the diversity in the first day of viremia of animals infected by contact versus the diversity in the sample of the donor the most likely day of transmission. Additionally, for the transmission by contact cases (5 animals) the frequency of each nucleotide in each position in the donor and the recipient were compared. With this it could be estimated what nucleotides increased or decreased their frequency in the transmission event. Frequency changes above 4.9% were arbitrarily considered relevant.

Analysis of molecular variance (AMOVA) in partially immune animals

In the case of partially immune animals, it was considered key to identify nucleotide positions that could be differentially selected in animals with longer or shorter viremias. Therefore, firstly animals were grouped according to the viremia as stated before. Then, the average frequencies of each nucleotide per position and group were compared using AMOVA in Arlequin ver 3.5.2.2 [44]. Positions presenting a Fct>0.05 were considered for further analysis.

Screening of potential changes in the amino acid composition in the viral proteins

For all cases (transmission to naïve or to immune animals), the resulting reads were ordered to represent the different viral proteins known. Once this was done, for the positions showing changes above the considered threshold for the frequencies of the nucleotides, the potential changes in codons were analysed.

In a first step, each change potentially causing the arising or the increasing of a heteronymous codon was annotated in the corresponding domain of the protein if known. Then, by bibliographic review, the affected positions were assessed for a known function. In addition, the changes in the protein structure were evaluated using SWISS-MODEL (https://swissmodel.expasy.org/repository/) and the potential modification in the charge of the protein or site were evaluated.

Acknowledgements

References

1. Gutiérrez S, Michalakis Y, Blanc S. Virus population bottlenecks during within-host progression and host-to-host transmission. Curr Opin Virol. 2012 Oct;2(5):546-55.

2. Bull RA, Luciani F, McElroy K, Gaudieri S, Pham ST, Chopra A, et al. Sequential bottlenecks drive viral evolution in early acute hepatitis C virus infection. PLoS Pathog. 2011 Sep;7(9):e1002243.

3. Sobel Leonard A, McClain MT, Smith GJ, Wentworth DE, Halpin RA, Lin X, et al. Deep Sequencing of Influenza A Virus from a Human Challenge Study Reveals a Selective Bottleneck and Only Limited Intrahost Genetic Diversification. J Virol. 2016 Nov 28;90(24):11247-11258.

4. Tully DC, Ogilvie CB, Batorsky RE, Bean DJ, Power KA, Ghebremichael M, et al. Differences in the Selection Bottleneck between Modes of Sexual Transmission Influence the Genetic Composition of the HIV-1 Founder Virus. PLoS Pathog. 2016 May 10;12(5):e1005619.

5. von Hahn T, Yoon JC, Alter H, Rice CM, Rehermann B, Balfe P, McKeating JA. Hepatitis C virus continuously escapes from neutralizing antibody and T-cell responses during chronic infection in vivo. Gastroenterology. 2007 Feb;132(2):667-78.

6. Díaz-Muñoz SL, Sanjuán R, West S. Sociovirology: Conflict, Cooperation, and Communication among Viruses. Cell Host Microbe. 2017 Oct 11;22(4):437-441.

7. Skums P, Bunimovich L, Khudyakov Y. Antigenic cooperation among intrahost HCV variants organized into a complex network of cross-immunoreactivity. Proc Natl Acad Sci U S A. 2015 May 26;112(21):6653-8.

8. Forsberg R. Divergence time of porcine reproductive and respiratory syndrome virus subtypes. Mol Biol Evol. 2005 Nov;22(11):2131-4.

9. Forsberg R, Oleksiewicz MB, Petersen AM, Hein J, Bøtner A, Storgaard T. A molecular clock dates the common ancestor of European-type porcine reproductive and respiratory syndrome virus at more than 10 years before the emergence of disease. Virology. 2001 Oct 25;289(2):174-9.

10. Pileri E, Gibert E, Martín-Valls GE, Nofrarias M, López-Soria S, Martín M, et al. Transmission of Porcine reproductive and respiratory syndrome virus 1 to and from vaccinated pigs in a one-to-one model. Vet Microbiol. 2017 Mar;201:18-25.

386 11. Rose N, Renson P, Andraud M, Paboeuf F, Le Potier MF, Bourry O. Porcine reproductive and
387 respiratory syndrome virus (PRRSv) modified-live vaccine reduces virus transmission in
388 experimental conditions. Vaccine. 2015 May 15;33(21):2493-9.

389 12. Díaz I, Darwich L, Pappaterra G, Pujols J, Mateu E. Immune responses of pigs after
390 experimental infection with a European strain of Porcine reproductive and respiratory syndrome
391 virus. J Gen Virol. 2005 Jul;86(Pt 7):1943-51.

392 13. Meier WA, Galeota J, Osorio FA, Husmann RJ, Schnitzlein WM, Zuckermann FA. Gradual
393 development of the interferon-gamma response of swine to porcine reproductive and
394 respiratory syndrome virus infection or vaccination. Virology. 2003 Apr 25;309(1):18-31.

395 14. Lopez OJ, Osorio FA. Role of neutralizing antibodies in PRRSV protective immunity. Vet
396 Immunol Immunopathol. 2004 Dec 8;102(3):155-63.

397 15. Martínez-Lobo FJ, Díez-Fuertes F, Simarro I, Castro JM, Prieto C. Porcine Reproductive and
398 Respiratory Syndrome Virus isolates differ in their susceptibility to neutralization. Vaccine. 2011
399 Sep 16;29(40):6928-40.

400 16. Díaz I, Gimeno M, Darwich L, Navarro N, Kuzemtseva L, López S, et al. Characterization of
401 homologous and heterologous adaptive immune responses in porcine reproductive and
402 respiratory syndrome virus infection. Vet Res. 2012 Apr 19;43:30.

403 17. Oleksiewicz MB, Bøtner A, Toft P, Normann P, Storgaard T. Epitope mapping porcine
404 reproductive and respiratory syndrome virus by phage display: the nsp2 fragment of the
405 replicase polyprotein contains a cluster of B-cell epitopes. J Virol. 2001 Apr;75(7):3277-90.

406 18. Burgara-Estrella A, Díaz I, Rodríguez-Gómez IM, Essler SE, Hernández J, Mateu E. Predicted
407 peptides from non-structural proteins of porcine reproductive and respiratory syndrome virus
408 are able to induce IFN-γ and IL-10. Viruses. 2013 Feb 11;5(2):663-77.

409 19. de Lima M, Pattnaik AK, Flores EF, Osorio FA. Serologic marker candidates identified among
410 B-cell linear epitopes of Nsp2 and structural proteins of a North American strain of porcine
411 reproductive and respiratory syndrome virus. Virology. 2006 Sep 30;353(2):410-21.

412 20. Oleksiewicz MB, Bøtner A, Normann P. Porcine B-cells recognize epitopes that are conserved
413 between the structural proteins of American- and European-type porcine reproductive and
414 respiratory syndrome virus. J Gen Virol. 2002 Jun;83(Pt 6):1407-18.

415 21. Di Giallonardo F, Zagordi O, Duport Y, Leemann C, Joos B, Künzli-Gontarczyk M, et al. Next-
416 generation sequencing of HIV-1 RNA genomes: determination of error rates and minimizing
417 artificial recombination. PLoS One. 2013 Sep 18;8(9):e74249.

418 22. Beerenwinkel N, Günthard HF, Roth V, Metzner KJ. Challenges and opportunities in
419 estimating viral genetic diversity from next-generation sequencing data. Front Microbiol. 2012
420 Sep 11;3:329.

421 23. Carlson JM, Schaefer M, Monaco DC, Batorsky R, Claiborne DT, Prince J, et al. HIV
422 transmission. Selection bias at the heterosexual HIV-1 transmission bottleneck. Science. 2014
423 Jul 11;345(6193):1254031.

424 24. da Silva RA, de Carvalho IM, de Matos RP, Yamasaki LH, Bittar C, Rahal P, Jardim AC. Evidence
425 of bottleneck effect on hepatitis C virus transmission between a couple under interferon based
426 therapy. Infect Genet Evol. 2017 Jan;47:87-93.

427 25. Li H, Stoddard MB, Wang S, Giorgi EE, Blair LM, Learn GH, et al. Single-Genome Sequencing
428 of Hepatitis C Virus in Donor-Recipient Pairs Distinguishes Modes and Models of Virus
429 Transmission and Early Diversification. J Virol. 2015 Oct 14;90(1):152-66.

430 26. Shen C, Gupta P, Xu X, Sanyal A, Rinaldo C, Seaberg E, Margolick JB, et al. Transmission and
431 evolution of hepatitis C virus in HCV seroconverters in HIV infected subjects. Virology. 2014 Jan
432 20;449:339-49.

433 27. Ma D, Jasinska AJ, Feyertag F, Wijewardana V, Kristoff J, He T, et al. Factors associated with
434 siman immunodeficiency virus transmission in a natural African nonhuman primate host in the
435 wild. J Virol. 2014 May;88(10):5687-705.

436 28. Delputte PL, Van Breedam W, Delrue I, Oetke C, Crocker PR, Nauwynck HJ. Porcine arterivirus
437 attachment to the macrophage-specific receptor sialoadhesin is dependent on the sialic acid-
438 binding activity of the N-terminal immunoglobulin domain of sialoadhesin. J Virol. 2007
439 Sep;81(17):9546-50.

440 29. Wissink EH, van Wijk HA, Kroese MV, Weiland E, Meulenberg JJ, Rottier PJ, van Rijn PA. The
441 major envelope protein, GP5, of a European porcine reproductive and respiratory syndrome
442 virus contains a neutralization epitope in its N-terminal ectodomain. J Gen Virol. 2003 Jun;84(Pt
443 6):1535-43.

444 30. Loving CL, Osorio FA, Murtaugh MP, Zuckermann FA. Innate and adaptive immunity against
445 Porcine Reproductive and Respiratory Syndrome Virus. Vet Immunol Immunopathol. 2015 Sep
446 15;167(1-2):1-14.

447 31. Das PB, Dinh PX, Ansari IH, de Lima M, Osorio FA, Pattnaik AK. The minor envelope
448 glycoproteins GP2a and GP4 of porcine reproductive and respiratory syndrome virus interact
449 with the receptor CD163. J Virol. 2010 Feb;84(4):1731-40.

450 32. Van Gorp H, Van Breedam W, Van Doorsselaere J, Delputte PL, Nauwynck HJ. Identification
451 of the CD163 protein domains involved in infection of the porcine reproductive and respiratory
452 syndrome virus. J Virol. 2010 Mar;84(6):3101-5.

453 33. Whitworth KM, Rowland RR, Ewen CL, Trible BR, Kerrigan MA, Cino-Ozuna AG. Gene-edited
454 pigs are protected from porcine reproductive and respiratory syndrome virus. Nat Biotechnol.
455 2016 Jan;34(1):20-2.

456 34. Dokland T. The structural biology of PRRSV. Virus Res. 2010 Dec;154(1-2):86-97.

457 35. Delputte PL, Vanderheijden N, Nauwynck HJ, Pensaert MB. Involvement of the matrix protein
458 in attachment of porcine reproductive and respiratory syndrome virus to a heparinlike receptor
459 on porcine alveolar macrophages. J Virol. 2002 May;76(9):4312-20.

460 36. Correas I, Osorio FA, Steffen D, Pattnaik AK, Vu HLX. Cross reactivity of immune responses to
461 porcine reproductive and respiratory syndrome virus infection. Vaccine. 2017 Feb 1;35(5):782-
462 788.

463 37. Parida R, Choi IS, Peterson DA, Pattnaik AK, Laegreid W, Zuckermann FA, Osorio FA. Location
464 of T-cell epitopes in nonstructural proteins 9 and 10 of type-II porcine reproductive and
465 respiratory syndrome virus. Virus Res. 2012 Oct;169(1):13-21.

466 38. Nogales A, Galán C, Márquez-Jurado S, García-Gallo M, Kremer L, Enjuanes L, Almazán F.
467 Immunogenic characterization and epitope mapping of transmissible gastroenteritis virus RNA
468 dependent RNA polymerase. J Virol Methods. 2011 Jul;175(1):7-13.

469 39. Sharmin R, Islam AB. A highly conserved WDYPKCDRA epitope in the RNA directed RNA
470 polymerase of human coronaviruses can be used as epitope-based universal vaccine design.
471 BMC Bioinformatics. 2014 May 29;15:161.

472 40. Pileri E, Gibert E, Soldevila F, García-Saenz A, Pujols J, Diaz I, et al. Vaccination with a
473 genotype 1 modified live vaccine against porcine reproductive and respiratory syndrome virus
474 significantly reduces viremia, viral shedding and transmission of the virus in a quasi-natural
475 experimental model. Vet Microbiol. 2015 Jan 30;175(1):7-16.

476 41. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data.
477 Bioinformatics. 2014 Aug 1;30(15):2114-20.

478 42. Li H, Durbin R. Fast and accurate long-read alignment with Burrows-Wheeler transform.
479 Bioinformatics. 2010 Mar 1;26(5):589-95.

480 43. Rozas J, Ferrer-Mata A, Sánchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE,
481 Sánchez-Gracia A. DnaSP 6: DNA Sequence Polymorphism Analysis of Large Data Sets. Mol Biol
482 Evol. 2017 Dec 1;34(12):3299-3302.

483 44. Excoffier L, Lischer HE. Arlequin suite ver 3.5: a new series of programs to perform population
484 genetics analyses under Linux and Windows. Mol Ecol Resour. 2010 May;10(3):564-7.

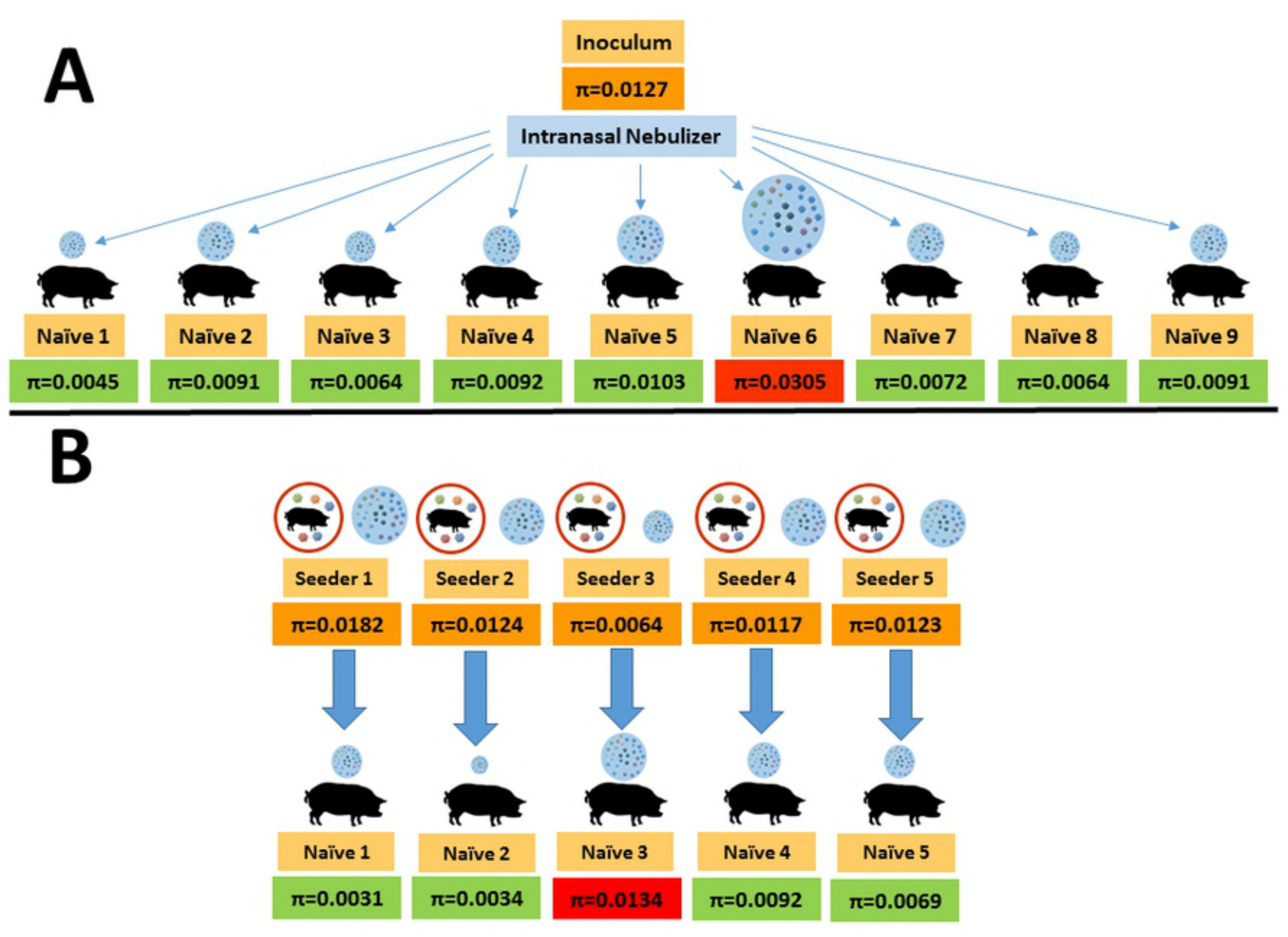
485 **Figure Captions**

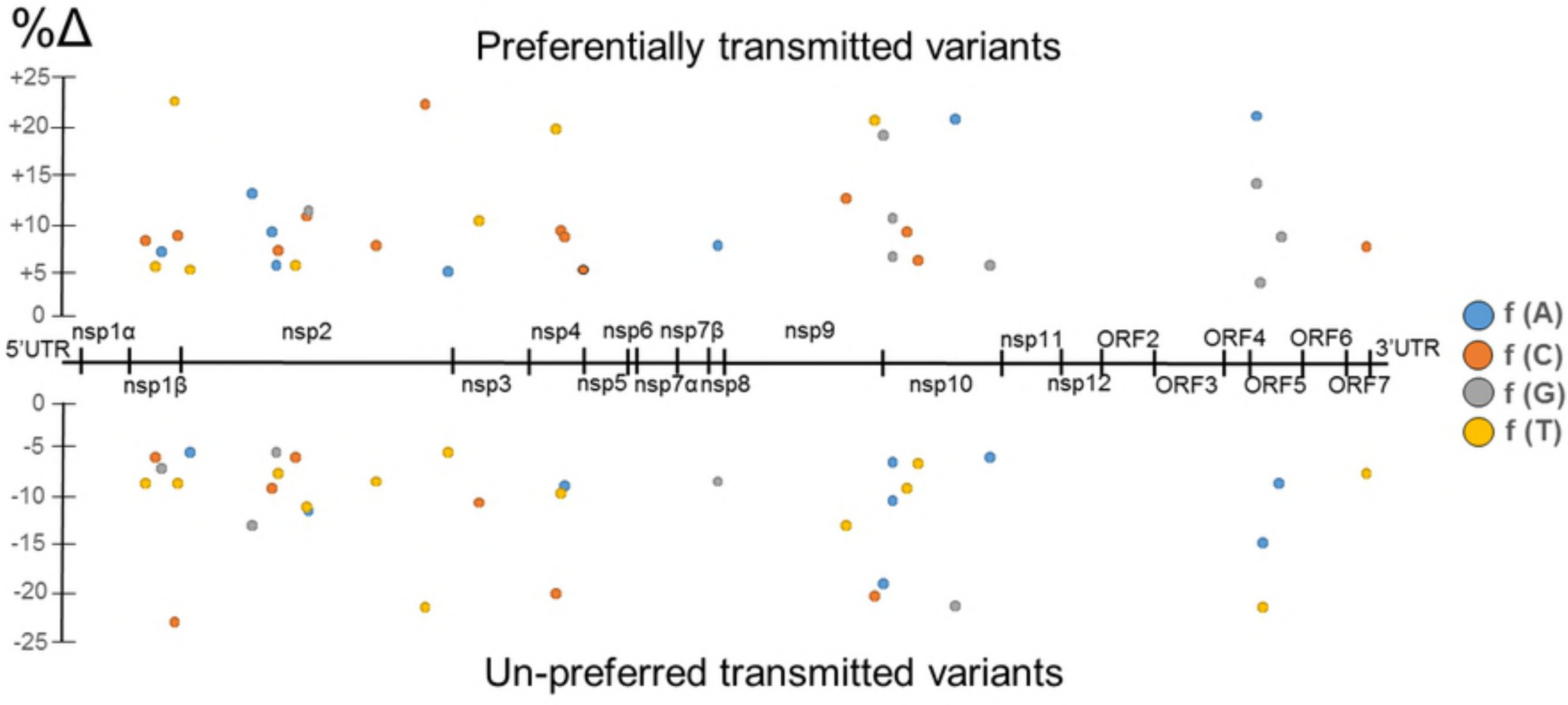
486 **Figure 1.** Summary of the 14 transmission events studied: **A**, intranasal inoculation of 9 naïve
487 pigs using a nebulizer; **B**, experimental infection in a 1:1 basis of 5 non-vaccinated naïve pigs
488 from seeders. Nucleotide diversity (π) estimations of the donor population (orange boxes) and
489 the founder variants, within green boxes if the global diversity decreases, in red if an increase is
490 reported.

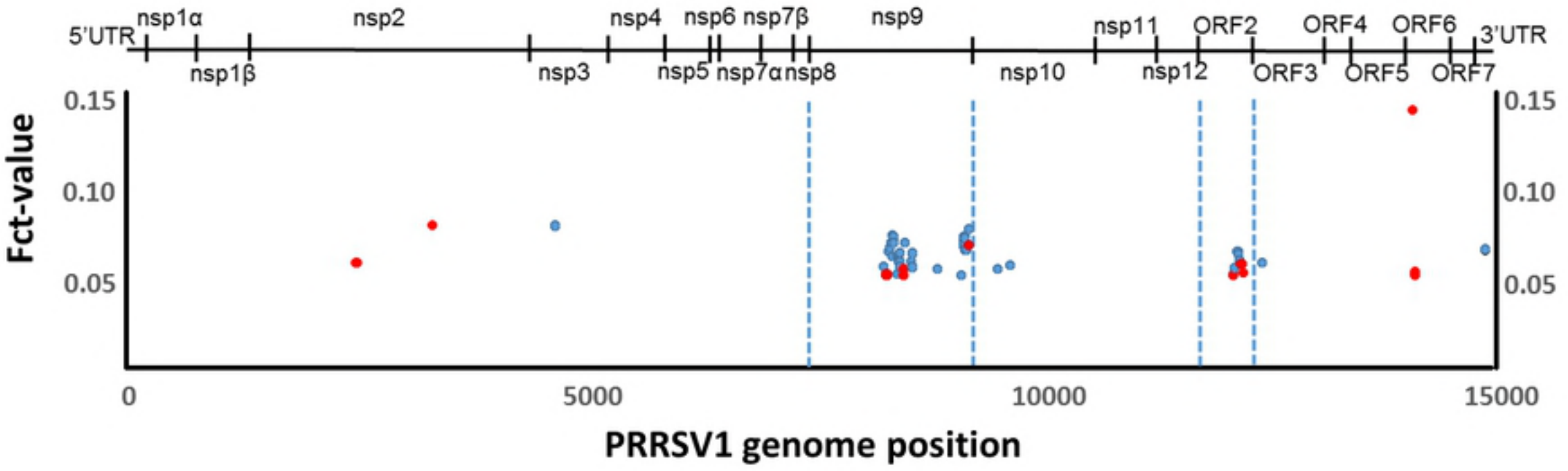
491 **Figure 2.** Mean percentage of variation in nucleotide frequencies (only positions with variations
492 larger than 5% are shown), along PRRSV1 genome, of the preferred or un-preferred transmitted
493 variants identified, for the transmission events between infected and naïve pigs studied (Fig.1B).

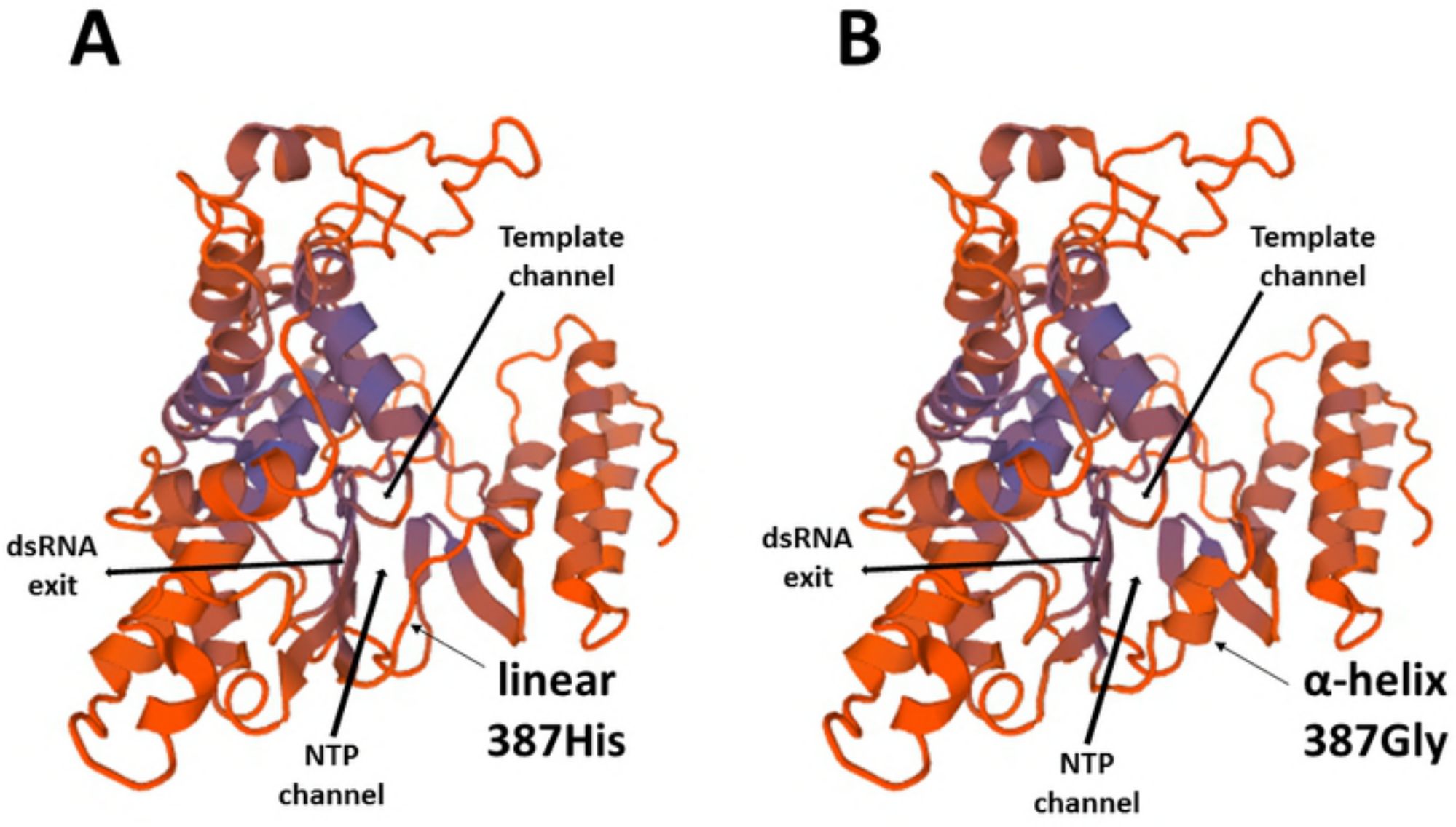
494 **Figure 3.** Location of the nucleotide positions along the PRRSV1 genome showing differences
495 (calculated as Fct results, only values >0.05 are shown) between two groups of vaccinated
496 animals developing short- (n=7) or long-viremia (n=4) against PRRSV1 infection.

497 **Figure 4.** 3D-structures of the PRRSV1 RdRp based on the translated sequences of nsp9 from the
498 viral quasi-species of the inoculum (A), long- (A) and short- (B) viremia pigs. The NTP and the
499 template channels, the dsRNA exit, as well as the amino acid residue 387 are indicated.


A
Inoculum
π=0.0127
Intranasal Nebulizer
Naïve 1
π=0.0045
Naïve 2
π=0.0091
Naïve 3
π=0.0064
Naïve 4
π=0.0092
Naïve 5
π=0.0103
Naïve 6
π=0.0305
Naïve 7
π=0.0072
Naïve 8
π=0.0064
Naïve 9
π=0.0091
B
Seeder 1
π=0.0182
Seeder 2
π=0.0124
Seeder 3
π=0.0064
Seeder 4
π=0.0117
Seeder 5
π=0.0123
Naïve 1
π=0.0031
Naïve 2
π=0.0034
Naïve 3
π=0.0134
Naïve 4
π=0.0092
Naïve 5
π=0.0069


%Δ
Preferentially transmitted variants
+25
+20
+15
+10
+5
0
5'UTR
nsp1α
nsp1β
nsp2
nsp3
nsp4
nsp5
nsp6
nsp7α
nsp7β
nsp8
nsp9
nsp10
nsp11
nsp12
ORF2
ORF3
ORF4
ORF5
ORF6
ORF7
3'UTR
f (A)
f (C)
f (G)
f (T)
0
-5
-10
-15
-20
-25
Un-preferred transmitted variants


5'UTR
nsp1α
nsp1β
nsp2
nsp3
nsp4
nsp5
nsp6
nsp7α
nsp7β
nsp8
nsp9
nsp10
nsp11
nsp12
ORF2
ORF3
ORF4
ORF5
ORF6
ORF7
3'UTR
Fct-value
0.15
0.10
0.05
0
5000
10000
15000
PRRSV1 genome position


A
Template
channel
dsRNA
exit
NTP
channel
linear
387His
B
Template
channel
dsRNA
exit
NTP
channel
α-helix
387Gly