

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

3 Short title: PRRSV1 quasi-species transmission and variation

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22 **Abstract**

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

34

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.

44 **Introduction**

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

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

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

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

82 Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically
83 important disease of pigs. It is caused by PRRS virus (PRRSV), a positive-sense, single-stranded
84 RNA virus in the *Arteriviridae* family within the order *Nidovirales*, exhibiting one of the highest
85 substitution rates observed [8, 9]. Experimental models to study PRRSV are well known and have
86 been applied to study transmission [10, 11]. Interestingly, the immune response against PRRSV
87 is unusual, since neutralizing antibodies appear late and cell-mediated immunity has an erratic
88 course for weeks. However, after several weeks of viremia, the virus is confined to the lymphoid
89 tissue and eventually cleared [12, 13]. Pre-formed neutralizing antibodies may protect against
90 the homologous infection in a dose dependent way [14], although that heterologous protection
91 cannot be foreseen [15]. Besides, there is a large individual variation in the immune response
92 [16]. As a result, when a vaccinated animal is challenged, viremia usually develops, but generally
93 of lesser duration than in a naïve animal.

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

100 **Results**

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

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

109 *The characterization of PRRSV transmission events supports the existence of bottlenecks*

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

117 Taking advantage of the availability of serial samples during the virological course of each
118 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
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

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

129 *genome*

130 Mean frequency differences for each nucleotide in every genome position were calculated for
131 the transmission events where naïve acceptors were infected by seeder pigs (Figure 1B). For this
132 section we used only data from pigs infected by contact, since for the inoculated pigs (Figure
133 1A), the Porcine Alveolar Macrophage (PAM) propagated inoculum might have shown some
134 level of adaptation to the cell passages and this might have masked the true changes associated
135 with transmission fitness. Figure 2 shows the mean increase or decrease in the frequencies of
136 each nucleotide per position (only values larger than 5% are shown), between the quasi-species
137 of the transmissible population and the transmitted founders. An increase in the nucleotide
138 frequency was associated with a preferentially transmitted variant, while a decrease was
139 identified as an un-preferred transmitted one. Along the PRRSV1 genome, 65 variants in 32
140 positions were detected (32 preferred and 33 un-preferred). Nineteen nucleotide variants
141 produced synonymous changes, while 13 variants induced potential amino acid changes.

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

146 Next, we examined and related the heteronymous changes with the regions and known features
147 of the encoded proteins. The six amino acid changes detected within nsp2 fall in the two
148 hypervariable regions flanking the papain-like cysteine protease domain, with one change (Gln-
149 336-Lys) located in the B-Cell epitope site 3 proposed by [17] and a second one (Tyr-736-His)
150 falling in a non-conserved epitope inducing IFN- γ and IL-10 responses, as reported by [18]. In
151 nsp4, an Ile-142-Leu change is located in the middle β -barrel domain II of the main PRRSV
152 proteinase 3C-like protease, but the change did not result in any substantial change in the 3D
153 structure of the protein. The single change detected in nsp5 – Leu-32-Phe – was located in a
154 transmembrane domain. Regarding nsp10, 2 out of 3 amino acid changes identified in this
155 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 led 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

181 by the C-terminal domain. The comparison of the RdRp 3D structures between *SV* and *LV* groups
182 (inferred with SWISS-MODEL), indicated that the amino acid changes Lys-338-Arg and Lys-641-
183 Arg do not produce any evident structural change. On the contrary, the change Gly-387-His,
184 caused by nucleotide changes in the three positions of the codon, induced a structural change.
185 The presence of a 387Gly in *SV* group resulted in the formation of an α -helix between residues
186 384-388 and reduced the positive charge at the opposite side of the active centre of the protein
187 where Mg^{2+} molecules bind. (Figure 4).

188 In GP2, the three amino acid changes identified, namely Gln-146-Arg, Val-151-Ala and His-184-
189 Arg, fall in the ectodomain, slightly downstream the linear B-cell epitopes proposed by [19] and
190 [20]. The amino acid changes reported for this protein implied more positively charged residues
191 in *LV* compared to *SV* group.

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

195 **Discussion**

196 With the introduction of NGS technologies, the experimental analysis of viral genetic diversity
197 has changed dramatically. Due to its massively parallel approach, NGS generates millions of
198 reads that cover every nucleotide position from thousands to hundreds of thousands times.
199 Hence, low-frequency variants within viral quasi-species can be adequately detected and
200 characterized [21] and it is possible to see variations that, although significant, would remain
201 unnoticed using Sanger sequencing. However, the usefulness of NGS for viral diversity
202 estimations depends crucially on the quality of the sample and on the procedure to prepare it
203 [22]. In the present work we applied a tailor-made NGS method to characterize the diversity of
204 PRRSV quasi-species. The method omitted the PCR amplification step and used instead a singly
205 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 sialoadhesin 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.

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

237 The third objective of this work was to analyse the quasi-species differences between two
238 groups of partially immune pigs developing short and long viremia. In the present work, the
239 duration of the viremia was correlated with the titers of neutralizing antibodies against the virus
240 [10]. Therefore, beforehand, main changes were expected to be located in potential targets for
241 the antibodies. Neutralizing antibodies for PRRSV have been reported to be induced by GP2,
242 GP3, GP4, GP5 and M proteins, although immune-dominant epitopes are mainly located in N
243 and nsp2 [14, 30]. In the present case most of the changes in SV occurred in nsp9, followed by
244 ORF2 that encodes GP2 (Figure 3). The 3D modelling of nsp9 (RdRp, Figure 4) showed that the
245 introduction of a Gly-387-His after changes in the three nucleotides of the codon, produced a
246 change in the folding of the protein, from linear to α -helix. This change resulted in the absence
247 of a positively charged group opposite to the site of union of Mg^{2+} , and would probably cause a
248 modification in the electrostatic forces involved in the interaction with the NTP channel. It is
249 reasonable to think that such a change would affect the efficiency of RNA synthesis.

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

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

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

259 In the frame of animals with higher titers of neutralizing antibodies, as observed in the SV group
260 [10], the changes reported in GP2 and M could be understood as escape mutations. The lower
261 antibody titers in the LV group probably prevented those changes from being positively selected,
262 and the major variants present in the initial inoculum remained the commonest. Similarly, it was
263 proposed that T-cell responses contributed to partial levels of cross-protection in PRRSV, and
264 therefore, the changes observed in nsp9 could be seen as escape variants [36]. Potential T-cell
265 epitopes for PRRSV have been proposed for nsp9 [37], as well as for the RdRp of other
266 *Nidovirales* [38, 39]. The striking fact relates with the fact that those changes in nsp9, GP2 and
267 M would probably result in less efficient viral variants for, either interacting with the cell
268 receptor, or replicating. This scenario suggests that in animals with higher levels of immunity,
269 the variants escaping the immune system are not fit enough to maintain a viable quasi-species
270 cloud, and consequently, the viral population collapses. Accordingly, nsp9, GP2 and M would be
271 clear targets for new vaccine development.

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

278 **Materials and Methods**

279 *Animal experiment*

280 Samples used in the present study were obtained in the course of a previous experiment aimed
281 to determine the transmission of PRRSV 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

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

338 *Analysis of molecular variance (AMOVA) in partially immune animals*

339 In the case of partially immune animals, it was considered key to identify nucleotide positions
340 that could be differentially selected in animals with longer or shorter viremias. Therefore, firstly
341 animals were grouped according to the viremia as stated before. Then, the average frequencies
342 of each nucleotide per position and group were compared using AMOVA in Arlequin ver 3.5.2.2
343 [44]. Positions presenting a $F_{ct} > 0.05$ were considered for further analysis.

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

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

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

355 **Acknowledgements**

356

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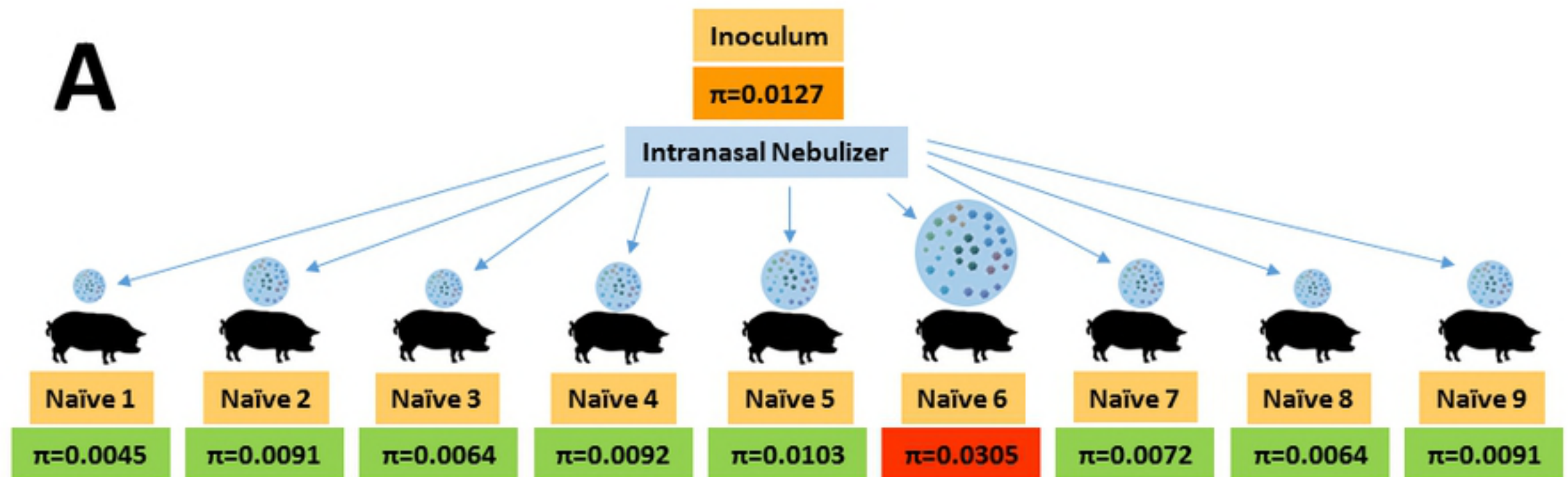
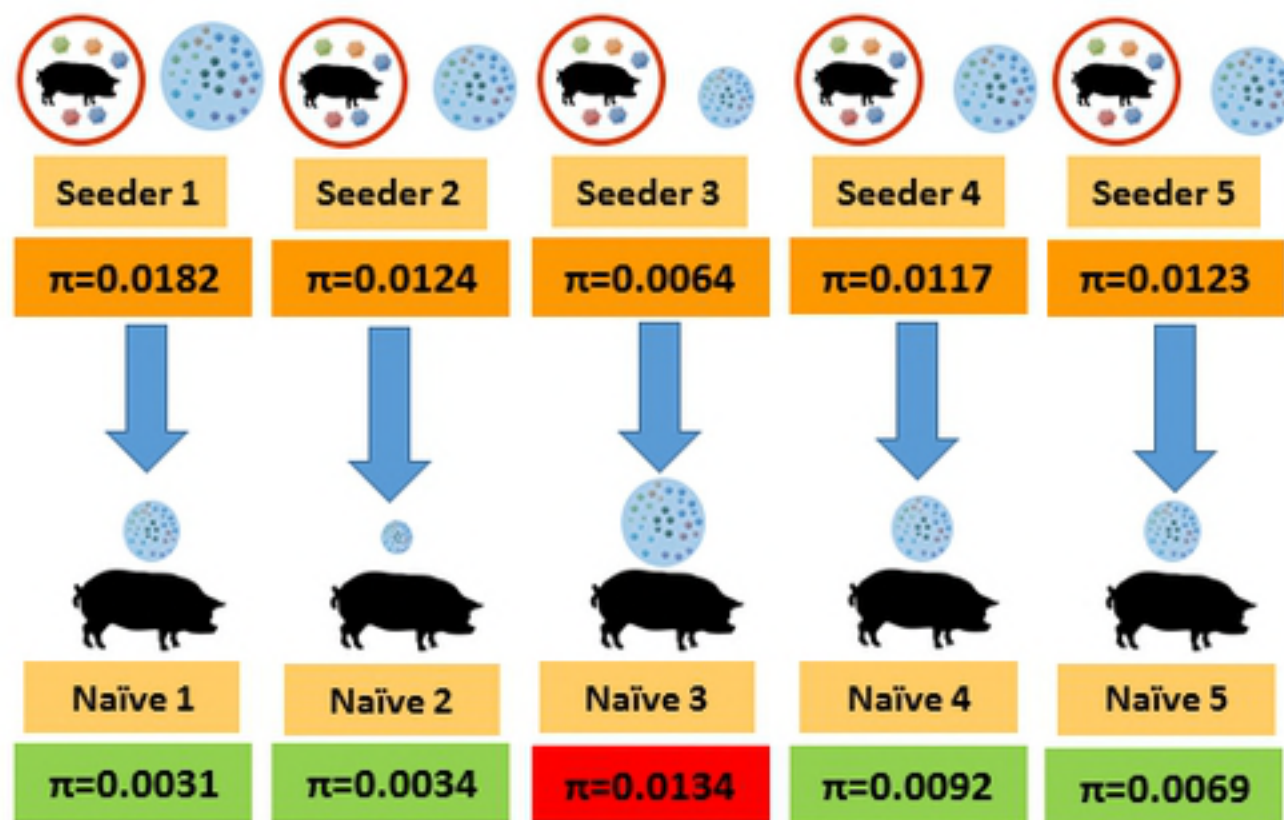
485 **Figure Captions**

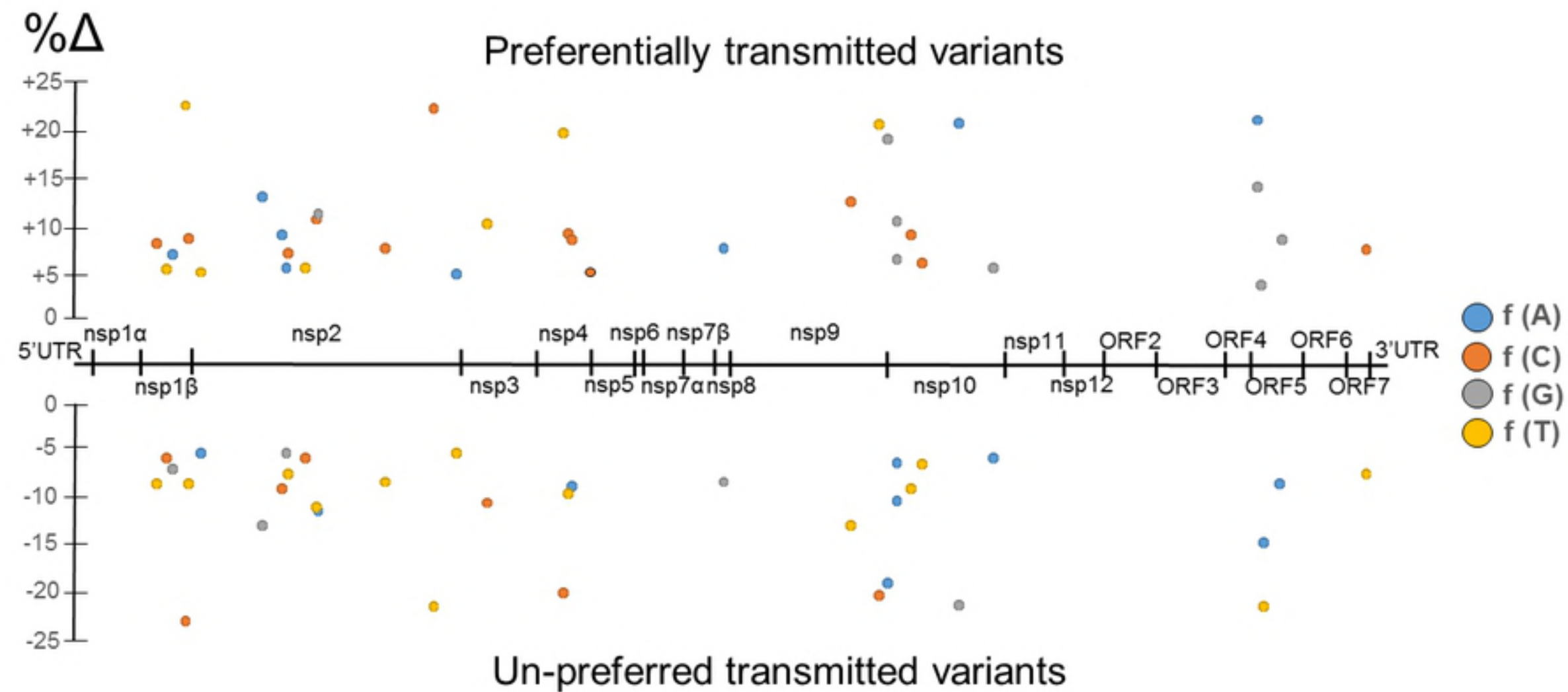
486 **Figure 1.** Summary of the 14 transmission events studied: **A**, intranasal inoculation of 9 naïve
487 pigs using a nebulizer; **B**, experimental infection in a 1:1 basis of 5 non-vaccinated naïve pigs
488 from seeders. Nucleotide diversity (π) estimations of the donor population (orange boxes)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

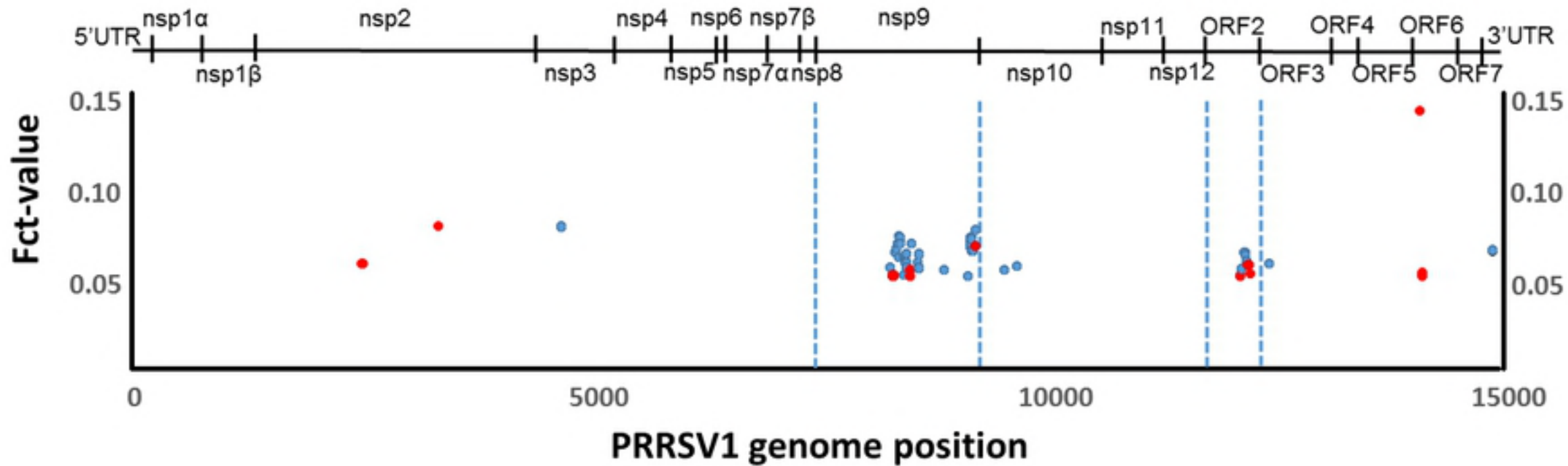
491 **Figure 2.** Mean percentage of variation in nucleotide frequencies (only positions with variations
492 larger than 5% are shown), along PRRSV1 genome, of the preferred or un-preferred transmitted
493 variants identified, for the transmission events between infected and naïve pigs studied (Fig.1B).

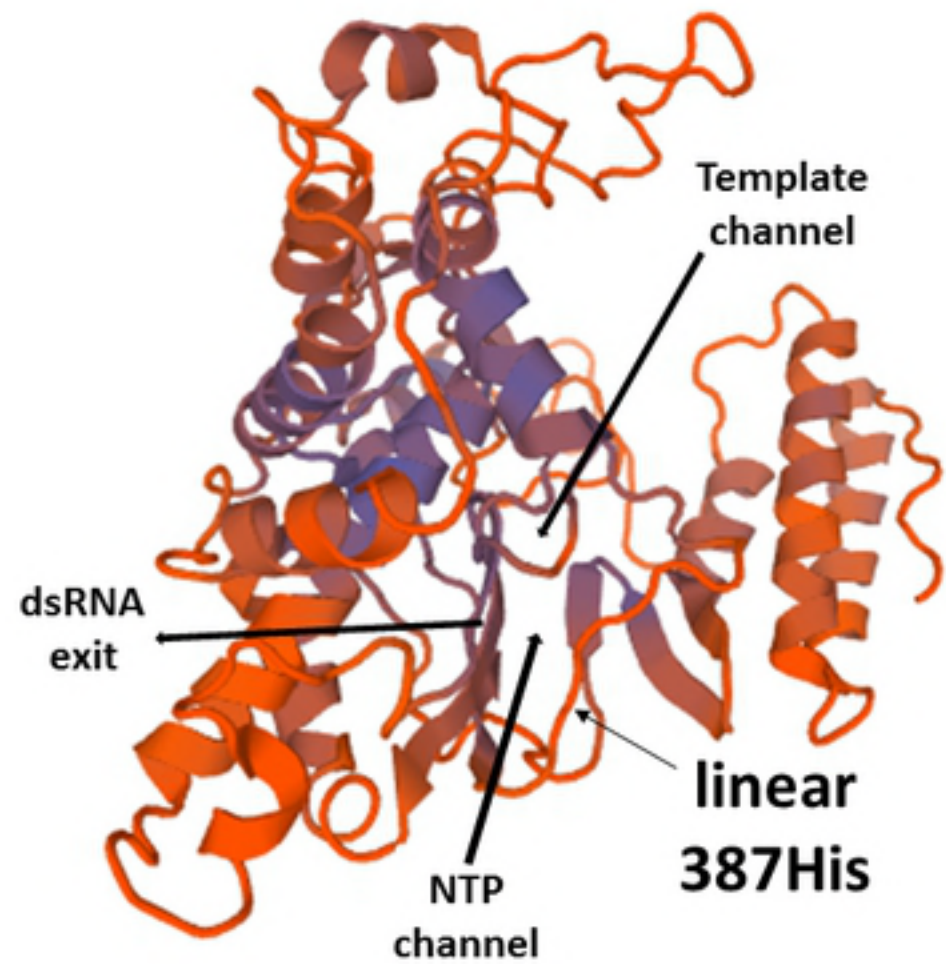
494 **Figure 3.** Location of the nucleotide positions along the PRRSV1 genome showing differences
495 (calculated as Fct results, only values >0.05 are shown) between two groups of vaccinated
496 animals developing short- (n=7) or long-viremia (n=4) against PRRSV1 infection.

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**B**





A**B**