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1 **Genome sequences of equine influenza A subtype H3N8 viruses by long read sequencing**
2 **and functional characterization of the PB1-F2 virulence factor of A/equine/Paris/1/2018**

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26 **Abstract**

27 Equine influenza virus (EIV) remains a persistent threat to equines, despite the availability of
28 vaccines. Currently, strategies to monitor the virus and prevent any potential vaccine failure
29 revolve around serological assays, RT-qPCR amplification, and sequencing the viral
30 hemagglutinin (HA) and neuraminidase (NA) genes. These approaches overlook the
31 contribution of other viral proteins in driving virulence. This study assesses the potential of
32 long-read nanopore sequencing for swift and precise sequencing of circulating equine influenza
33 viruses. To this end, two French Florida Clade 1 strains, including the one circulating in winter
34 2018-2019 exhibiting more pronounced pathogenicity than usual, as well as the two currently
35 used OIE-recommended vaccine strains, were sequenced. Our results demonstrated the
36 reliability of this sequencing method in generating accurate sequences. Sequence analysis of
37 HA revealed a subtle antigenic drift in the French EIV strains, with specific substitutions, such
38 as T163I in A/equine/Paris/1/2018 and the N188T mutation in post-2015 strains; both
39 substitutions were located in antigenic site B. Antigenic site E exhibited modifications in post-
40 2018 strains, with the N63D substitution. Segment 2 sequencing also revealed that the
41 A/equine/Paris/1/2018 strain encodes a longer variant of the PB1-F2 protein when compared to
42 other Florida clade 1 strains (90 amino acids long versus 81 amino acids long). Further
43 biological and biochemistry assays demonstrated that this PB1-F2 variant has enhanced
44 abilities to abolish the mitochondrial membrane potential $\Delta\Psi_m$ and permeabilize synthetic
45 membranes. Altogether, our results highlight the interest in rapidly characterizing the complete
46 genome of circulating strains with next-generation sequencing technologies to adapt vaccines
47 and identify specific virulence markers of EIV.

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51 **Introduction**

52 Equine influenza (EI) is a highly contagious respiratory disease affecting horses, with
53 significant economic repercussions on the global equine industry [1–4]. Its widespread
54 transmission is facilitated by the international transport of horses, primarily for competition and
55 breeding purposes [5, 6]. Common clinical manifestations of EI infection in naïve and
56 unprotected animals include pyrexia, persistent cough, serous nasal discharge, dyspnea, muscle
57 pain or weakness, lethargy, anorexia, and often complications arising from secondary bacterial
58 infections [7, 8]. Although rarely fatal on its own, EI can lead to secondary bacterial infections
59 in the respiratory tract and lungs, exacerbating the clinical condition of affected horses [4, 8].

60 Equine influenza virus (EIV), which is the causal agent of EI, is an influenza type A virus
61 belonging to the *Orthomyxovirus* genus within the *Orthomyxoviridae* family. Currently, EI is
62 known to be caused by only two primary virus subtypes: H3N8 and H7N7, with the latter
63 remaining undetected since the 1970s [9]. The H3N8 subtype emerged in 1963 [10] in the
64 Americas and has since spread globally, continuing to trigger epizootic events [2, 3, 11–13]. In
65 the 1980s, H3N8 further diverged into American and Eurasian lineages [14]. The American
66 lineage subsequently branched into the Kentucky, South American, and Florida sublineages
67 [15]. The Florida sublineage underwent additional evolution in the early 2000s, resulting in two
68 subtypes: Florida sublineage clade 1 (FC1) and Florida sublineage clade 2 (FC2) [16]. FC1
69 predominantly circulated in the Americas, while FC2 prevailed in Europe. However, this
70 pattern shifted with the 2009 outbreak of an FC1 strain in Europe [17, 18]. Subsequently, EIV
71 FC1 caused an outbreak of an unprecedented scale between late 2018 and 2019 in Europe [12,
72 19], with 53 outbreaks reported in France, 228 in the United Kingdom, and approximately 80
73 in Ireland [20, 21]. During the 2018 outbreak, vaccination coverage was substantial in France
74 [20]. The vaccines used during these outbreaks are still considered effective by the World
75 Organization for Animal Health Expert Surveillance Panel (OIE ESP) [20–22].

76 Currently, most diagnostic tests for EIV rely on detecting viral antigens or RT-qPCR
77 amplification of viral nucleic acids obtained from nasal swab samples. These two approaches
78 have distinct trade-offs: antigen testing is swift but has limited sensitivity, while RT-qPCR is
79 more time-consuming but offers higher sensitivity. Moreover, data generated by these methods
80 have limitations in providing insights into epidemiological links and vaccine effectiveness. In
81 most cases, sequencing of the viral strains is performed posteriorly by Sanger sequencing using
82 several segment-specific primers [23]. This technique is efficient but very time-consuming, and
83 multiplexing is not possible. Therefore, there is a need to develop new diagnostic tools that
84 combine speed, sensitivity, ability to detect coinfections, and comprehensive genome sequence
85 information. Such methods are vital for effective health management strategies, including the
86 identification of potential new virulence factors and the precise design of vaccines.

87 In this study, our objective was to genetically characterize the equine influenza H3N8 viruses
88 circulating in France during the winters of 2009 and 2018 and, more specifically, to identify
89 and characterize potential virulence determinants and antigenicity through whole-genome
90 sequencing. Therefore, we used MinION long-read sequencing technology, which offers rapid
91 sequencing and multiplex barcoding [24–27]. The viral strains A/equine/Beuvron-en-
92 Auge/2/2009 and A/equine/Paris/1/2018, along with the OIE-recommended vaccine strains
93 A/equine/Richmond/1/2007 and A/equine/South Africa/4/2003, were sequenced. Our results
94 suggest that the accessory protein PB1-F2 may contribute to the virulence of the
95 A/equine/Paris/1/2018 strain.

96

97 **Results**

98 **Full-length genome sequencing strategy**

99 The four selected EIV strains A/equine/Beuvron-en-Auge/2/2009 and A/equine/Paris/1/2018
100 as well as the OIE-recommended vaccine strains A/equine/Richmond/1/2007 and

101 A/equine/South Africa/4/2003 were used to obtain complete amplicon sequences using the
 102 long-read sequencing technology developed by Oxford Nanopore Technology. The workflow
 103 used is described in **Fig. 1**. Direct RNA sequencing was carried out using the A/equine/South
 104 Africa/4/2003 strain to evaluate the relative sensitivity and accuracy of this approach (data not
 105 shown).
 106

		South Africa	Richmond	Beuvron	Paris
Total reads		515 999	519 851	976 418	582 633
Filtered reads (Q>10; Size>600 bp)	Reads number	158 077	173 495	420 018	189 296
	Average length (nt)	1 291	1 132	974	1 215
	Average quality	22.5	22.6	22.1	22.6
Mapped reads	Total reads mapped	157 979	173 393	419 899	188 939
	% reads mapped	99.94%	99.94%	99.97%	99.81%
	Segment1 (PB2)	20 197	27 727	80 078	26 715
	Segment2 (PB1, PB1-F2)	18 731	26 059	135 601	26 609
	Segment3 (PA)	19 554	24 642	31 455	33 794
	Segment4 (HA)	17 898	21 245	35 347	28 913
	Segment5 (NP)	27 086	16 715	19 096	20 082
	Segment6 (NA)	23 350	21 141	29 387	21 860
	Segment7 (M1-M2)	26 425	35 844	54 494	35 756
	Segment8 (NS1, NEP)	23 634	17 339	47 279	25 569

107 **Table 1. Sequencing statistics.** Detailed sequencing statistics obtained after demultiplexing
 108 and size and quality filtering of the sequenced strains A/equine/South Africa/4/2003 (South
 109 Africa), A/equine/Richmond/1/2007 (Richmond), A/equine/Beuvron-en-Auge/2/2009
 110 (Beuvron), and A/equine/Paris/1/2018 (Paris).

111
 112 After size and quality filtering, a mean of 235 222 reads per strain with 158 077 reads for
 113 A/equine/South Africa/4/2003, 173 495 reads for A/equine/Richmond/1/2007, 189 296 for
 114 A/equine/Paris/1/2018 and 420 018 reads for A/equine/Beuvron-en-Auge/2/2009 were
 115 produced (detailed sequencing statistics in **Table 1**). The average read length was 1291 bp for

116 A/equine/South Africa/4/2003, 1132 bp for A/equine/Richmond/1/2007, 1215 bp for
117 A/equine/Paris/1/2018 and 974 bp for A/equine/Beuvron-en-Auge/2/2009. The average quality
118 (Phred score) for the four strains was Q=22. For the four strains, a mapping rate varying
119 between 99.81% and 99.97% with full coverage of the eight influenza genome segments was
120 obtained using the reference genome A/equine/Ohio/113461-1/2005.

121 The nucleotide sequences of the viral genomes of the four strains were compared to those of
122 A/equine/Ohio/113461-1/2005 (**Fig. 2, Supp Figs. 1 and 2**). No nucleotide discrepancies were
123 observed between the genome sequence generated by amplicons and direct RNA sequencing
124 of A/equine/South Africa/4/2003 (data not shown). A total of 538 substitutions for the four
125 strains were detected. The A/equine/Paris/1/2018 genome exhibited a higher number of
126 nucleotide substitutions (287 substitutions), particularly in the HA and NA segments, with 45
127 substitutions for each. Additionally, higher nucleotide sequence diversity was found in
128 segments 1 and 3, encoding RNA-polymerase (FluPol) subunits PB2 and PA, respectively, with
129 53 and 49 substitutions among them and 32 and 31 being specific to A/equine/Paris/1/2018.

130

131 **Phylogenetic analyses**

132 Individual phylogenetic trees were constructed for each of the eight segments, including
133 sequences from the literature. The accession numbers of the selected sequences are presented
134 in **Supp Table 1. Fig. 3** shows the analysis of complete HA and NA coding sequences. From
135 2011, the French isolates were present in both the FC1 and FC2 strains, with the
136 A/equine/Paris/1/2018 HA segment exhibiting a higher phylogenetic distance from the vaccine
137 strains. These observations for the HA gene were correlated with the complete NA sequence
138 analysis. **Fig. 4A-D** shows the phylogenetic trees of the four segments encoding the components
139 of the influenza ribonucleoprotein complex (with NP and FluPol subunits PA, PB1, and PB2).
140 While all the phylogenetic trees correlate well with those of the HA and NA segments, the PA

141 and PB1 subunits of A/equine/Saone-et-Loire/1/2015 exhibited a higher divergence than those
142 of the other viruses, possibly reflecting the mark of a reassortment event with these two
143 segments. In the same way, the phylogenetic trees carried out on segments 7 (M) and 8 (NS)
144 correlated well with the ones described above, with exceptions in segment 7 of the two FC2
145 viruses (A/equine/Jouars/4/2006 and A/equine/Newmarket/5/2003) that appear to segregate
146 with FC1 viruses and in segment 8 of two FC1 strains (A/equine/South Africa/4/2003 and
147 A/equine/Ohio/1/2003) that group with FC2 viruses (**Fig. 4E-F**).

148

149 **Analysis of HA amino acid alignment between circulating and ancestral viruses with** 150 **vaccine strains**

151 *The antigenic sites.* Five antigenic sites (A-E) have been previously defined on the
152 hemagglutinin of influenza viruses of the H3 type (**Fig. 5 and Supp Fig. 3**), [28–31]). **Fig. 5A**
153 shows a multiple alignment of amino acid sequences defining these antigenic sites on a
154 selection of equine H3N8 viruses. **Fig. 5B** highlights the positions of the antigenic sites on the
155 HA 3D structure. The recently circulating virus strains A/equine/Paris/1/2018 and
156 A/equine/Beuvron-en-Auge/2/2009 were included in the analysis, with viruses belonging to
157 FC1 and FC2 with representatives of French EIV strains and vaccine strains currently used in
158 France (A/equine/Ohio/1/2003 and A/equine/Richmond/1/2007). Relatively high stability of
159 the antigenic sites was observed for the FC1 and FC2 viruses over 40 years when compared to
160 the two viruses isolated in 1963, A/equine/Miami/1/1963 and A/equine/Uruguay/1/1963.
161 Among the 101 residues constituting the antigenic sites, only 19 and 18 substitutions were
162 identified in A/equine/Paris/1/2018 and A/equine/Saone-et-Loire/1/2015, respectively. When
163 compared with the currently used vaccine strains, only four substitutions (R62K, N63D, A138S
164 and N188T) between FC1 circulating strains and A/equine/Ohio/1/2003 and three (A144T,

165 T192K and Q197R) between FC2 strains A/equine/Saone-et-Loire/1/2015 and
166 A/equine/Richmond/1/2007 were identified.

167 When restricting the analysis to three predivergent strains (with two of the 1963 years, the date
168 of recognized emergence of H3N8 EIV), twelve amino acid substitutions occurred in the HA
169 antigenic sites, several of them being conserved in subsequent clusters (T48I, M121T, G137G,
170 E158G, S159N, T163I, A198E, and V242I). Others (E82G, G135S, D172N, and S199L) were
171 not conserved among representatives of circulating strains of FC1 and FC2 when they diverged
172 from 2003. Eurasian and American lineages (that emerged in the 1980s) displayed additional
173 common substitutions (P55S, G135R/T, R140K, D172K, T187S, N189Q, and V196I) that were
174 conserved in FC1 and FC2 circulating strains. Others (T48I, K156N, N189K, K207E, and
175 T212V) were only represented in these two lineages. Among them,
176 A/equine/Switzerland/173/1993 (Eurasian lineage) displayed additional specific substitutions
177 (V78D, K156N, I213R, and P273L). A/equine/Newmarket/1/1993 (American lineage) also
178 displayed a specific substitution (K193E). Concerning the FC1 and FC2 strains, T48M
179 appeared to be the unique substitution marking these two sublineages. Other conserved
180 substitutions (compared to the 1963 strains) were previously identified in the American lineage.
181 The S159 variant was found only in the A/equine/Miami/1/1963 strain, and the V78A
182 substitution is a hallmark of the FC1 strains when compared to other strains. As exemplified in
183 **Fig. 5C**, several specific substitutions represented in different FC1 strains are R62K, N63D,
184 A138S and N188T. For FC2 viruses, only one substitution in an antigenic site (A144T) was
185 observed between the vaccine strain (A/equine/Richmond/1/2007) and the A/equine/Saone-et-
186 Loire/1/2015 virus (**Fig. 5D**, [11]).

187 **The receptor binding site.** Because of the importance of receptor binding by HA in virus
188 transmission and cross-species barriers, the analysis was extended to residues associated with
189 binding to α 2,3-linked receptors (**Supp. Fig. 3**). These residues are present on two loops on

190 HA1, the 130-loop, the 220-loop, and the 190-helix [32, 33]. As expected, HA1 G225 and Q226
191 (220-loop), which are involved in receptor binding, are strictly conserved among all the strains
192 analyzed. E190 and K193 are highly conserved (with two exceptions, E190Q and K193E in
193 A/equine/Newmarket/1/1993). R135 and G137 (in the 130-loop and antigenic site A) exhibited
194 full conservation in FC1 and FC2. Amino acid substitutions in the two loops were also
195 identified in FC1 viruses (A138S and V223I).

196 ***The membrane fusion machinery.*** Two amino acid stretches in HA1 (a loop from residue 25
197 to 35) and HA2 (α-helix A between residues 367 and 384) constitute the fusion subdomain of
198 HA that governs the fusion between cell and viral membranes. A single amino acid substitution,
199 T30S, which was proposed to influence membrane fusion activity through local perturbation of
200 the interactions between these two stretches [32], was identified in all FC1 and FC2 viruses. At
201 position 379, a G379E substitution in several FC1 and FC2 viruses was observed. 3D structures
202 of the HA of a Eurasian virus and an FC2 virus show that the glycine marks a break of the α-
203 helix A [32], thus possibly modulating their fusion properties. The two HAs of the French
204 strains A/equine/Paris/1/2018 and A/equine/Beuvron-en-Auge/2/2009 have a Gly at position
205 379.

206 Additional substitutions that are not involved in antigenic sites, receptor binding, or the fusion
207 machinery are reported in **Supp. Table 2**.

208

209 **Analysis of NA amino acid alignment**

210 Fourteen substitutions were identified between A/equine/Paris/1/2018 and the vaccine strain
211 A/equine/Ohio/1/2003, seven in the stalk (A13T, N21S, V35A, G47E, T68I, I74M, R76K) and
212 seven in the head (V147I, R252K, D258N, R260K, S337N, G416E and T434K) (**Supp. Table**
213 **2** and **Supp Fig. 4**). **Fig. 6** shows the substitutions exposed on the surface of the head of NA,
214 one of them (V147I) located near the 150 loop of the active site [34].

215

216 **Comparison of the viral proteins of the replicative complex**

217 The amino acid sequences of the FluPol subunits (PA, PB1 and PB2) and NP of the two FC1
218 strains A/equine/Paris/1/2018 and A/equine/Beuvron-en-Auge/2/2009 were compared with
219 A/equine/Ohio/1/2003 and A/equine/Richmond/1/2007, the two OIE-recommended vaccine
220 strains representing FC1 and FC2, respectively (**Fig. 7**). A greater number of changes in the
221 EIV strain A/equine/Paris/1/2018 were identified in comparison to A/equine/Ohio/1/2003. This
222 strain from 2018 possesses eight amino acid substitutions in PA, one in PB1, nine in PB2, and
223 one in NP with A/equine/Ohio/1/2003. Some substitutions were also identified in
224 A/equine/Beuvron-en-Auge/2/2009, such as in PB2 I63V, I398V, V667 and V686I, in PB1
225 F94L, R584Q and K621R and in PA E237K and T354I. Twenty-two substitutions between
226 these two strains on the FluPol subunits and NP were also identified, exemplifying the
227 continuous accumulation of substitutions between 2009 and 2018 in FC1 strains. Twenty-one
228 substitutions between the two vaccine strains (isolated in 2003 and 2007) and three between
229 A/equine/Ohio/1/2003 and A/equine/South Africa/4/2003 were also observed.

230

231 **Comparison of M1, M2, NS1 and NEP proteins**

232 Although eleven substitutions were found (mainly accumulating in M1) between
233 A/equine/Richmond/1/2007 and A/equine/Ohio/1/2003, only ten substitutions were identified
234 between A/equine/Paris/1/2018 and A/equine/Ohio/1/2003 (four in NS1) (**Fig. 7**).

235

236 **PB1-F2**

237 The analysis of the gene product PB1-F2, encoded by a +1 reading frame shift of segment 2,
238 showed a large number of substitutions. PB1-F2 is an accessory (nonstructural) protein that
239 presents the highest percentage of substitutions, with twenty-two substitutions for the short

240 versions of PB1-F2 made of 81 amino acids. Interestingly, a stretch of nine residues was present
241 at the C-ter of PB1-F2 encoded by all the predivergent strains [35], but only in a single FC2
242 virus (A/equine/Saone-et-Loire/1/2015) and in four of the eleven FC1 strains analyzed,
243 suggesting that PB1-F2 functions in equine cells do not need these last amino acid stretches
244 (**Fig. 8**). While amino acids that have been described to be associated with pathogenicity (T51
245 and V56; [36]) are conserved among the analyzed strains, residues involved in the inflammatory
246 response (R75 and R79; [37]) are not systematically present. The S66N substitution was
247 identified in all the PB1-F2s analyzed, except those of the predivergent strains, possibly
248 marking a decrease in virulence [38, 39].

249

250 **Functional characterization of equine PB1-F2**

251 Although PB1-F2 is dispensable for virus replication, it plays significant roles in pathogenesis
252 by altering inflammatory responses, interfering with the host's innate immune response, and
253 promoting secondary bacterial infections [39–52]. In infected cells, variants of PB1-F2 target
254 mitochondria [42, 46, 53]. Recombinant PB1-F2 has been shown to destabilize and
255 permeabilize synthetic membranes [54–56]. To compare the respective properties of long (90-
256 amino acids long) versus short (81-amino acids long) forms of PB1-F2 of equine viruses,
257 plasmids encoding its A/equine/Paris/1/2018 and the A/equine/Ohio/1/2003 variants were
258 transfected, and their effects on mitochondrial activity were analyzed. **Fig. 9A** shows that the
259 expression of both forms of PB1-F2 resulted in the suppression or lowering of their
260 mitochondrial inner-membrane potential when compared to cells that did not express it,
261 according to a strong decrease in the MitoTracker staining in cells expressing PB1-F2.

262 To further compare the intrinsic properties of the two variants, a lipid vesicle permeabilization
263 assay was used with large unilamellar vesicles (LUVs) composed of synthetic lipid vesicles
264 mimicking the composition of the outer mitochondrial membrane (OMM) [57]. The two PB1-

265 F2 variants were incubated with LUVs containing a fluorescent soluble probe (ANTS) and its
266 quencher (DPX). The permeabilization of LUVs induced ANTS and DPX efflux, which
267 consequently resulted in dilution and dissociation of the fluorescent probe and its quencher in
268 the extravesicular milieu, as revealed by an increase in ANTS fluorescence. **Fig. 9B** shows that
269 both PB1-F2 variants induced permeabilization of the vesicles in a dose-dependent manner, and
270 the specific permeabilization activity of the A/equine/Paris/1/2018 PB1-F2 variant was twofold
271 higher than that of its homolog.

272

273 **Discussion**

274 **Whole-genome sequencing.** Whole-genome sequences of four equine influenza viruses were
275 produced using a long-read nanopore sequencer on amplicon RT-PCR products. In addition,
276 these sequences were compared to those obtained by the same sequencer using a direct RNA
277 sequencing library, which could be innovative for characterizing native viral RNA genomes.
278 We confirmed that direct RNA sequencing requires a large amount of RNA material, rendering
279 the accuracy of the sequencing difficult to control [58]. However, when carried out with
280 sufficient material, the two sequencing approaches fulfilled the needed standard for nucleotide
281 sequence determination.

282 **Phylogeny.** The phylogenetic trees of genomic segments confirmed that A/equine/Paris/1/2018
283 and A/equine/Beuvron-en-Auge/2/2009 belonged to the EIV H3N8 FC1 (**Fig. 3** and)11, 12,
284 20]) and did not allow the identification of possible segment reassortment events between EIVs.
285 With the integration in our analyses of viruses belonging to FC2, we possibly identified with
286 the phylogenetic tree of segment 7 the mark of a reassortment event in A/equine/Jouars/4/2006
287 and A/equine/Newmarket/5/2003 between clades 1 and 2 EIV (Fig. 4E). While these two
288 viruses group with FC2 viruses in segments 1-6 and 8, they appear to be more related to FC 1

289 viruses with their segment 7 sequences. Further investigations are needed to validate this
290 observation.

291 **Antigenicity.** Since 2010, the OIE-ESP has recommended the incorporation of representative
292 EIV strains from both FC1 and FC2 into EI vaccines. Comparison of HA sequences highlights
293 several substitutions between the French EIV strains and the OIE-recommended strain
294 A/equine/Ohio/1/2003 (FC1). The strain A/equine/Paris/1/2018 presents twenty-two
295 substitutions when compared to A/equine/Ohio/1/2003, five of which (A138S, T163I, N188T,
296 R62K, and N63D) are in antigenic sites (site A for the first residue, site B for the two following
297 residues and site E for the last two). The accumulation of these amino acid substitutions within
298 the antibody-binding sites in HA could be sufficient to lead to antigenic drift. We identified one
299 of these substitutions (T163I) only in A/equine/Paris/1/2018 when compared to FC1 and FC2
300 viruses. According to Wilson and Cox (1990) [59], four or five amino acid substitutions in two
301 separate antigenic sites should be sufficient for escape from preexisting immunity and lead to
302 vaccine failure for human influenza A viruses. For equine influenza A viruses, 10-16 amino
303 acid differences between outbreak and vaccine strains could lead to vaccine breakdown [30,
304 60]. These results confirm that an EIV clade 1 virus, A/equine/Ohio/1/2003, still constitutes an
305 efficient vaccine strain in recent EIV outbreaks, as previously shown in a large-scale serological
306 study [20]. A similar conclusion could be reached with circulating FC2 EIV and the OIE-
307 recommended vaccine strain A/equine/Richmond/2007, with only 4 substitutions identified in
308 the antigenic sites.

309 **Equine influenza markers.** Equine influenza H3N8 viruses represent a single genetic lineage
310 [61] resulting from the crossover of an avian influenza virus since its first isolation in 1963
311 [10]. The adaptation of avian influenza A virus to the equine host has been documented, and
312 several host-specific markers have been identified [61, 62]. Comparison between the FluPol,
313 M1, M2, NS1, and NEP sequences of A/equine/Paris/1/2018 and A/equine/Beuvron-en-

314 A/equine/Beuvron-en-Auge/2/2009 with representatives of earlier and FC2 strains shows a general conservation of
315 the equine-specific markers with some exceptions. In PB1, a reversion from the recent (since
316 1997) equine marker I114 was identified in A/equine/Paris/1/2018 (FC1) and A/equine/Saone-
317 et-Loire/1/2015 (FC2) to valine. Additionally, the F94L and K621R substitutions appeared
318 since 2005 in FC1 viruses only. In PA, reversion of the equine E237 to the avian K237 marker
319 has been observed for the most recent Fc1 strain (A/equine/Paris/1/2018) and since 2007 for
320 FC2 strains. This position pertains to a cluster of additional equine-specific markers (positions
321 213, 216, 217, 231, and 244). S409N substitution was also revealed in A/equine/Paris/1/2018
322 and A/equine/Beuvron-en-Auge/2/2009, confirming a previously recognized mammal
323 adaptation marker [63] in FC1 viruses [62]. In PB2, the I398V substitution was identified in
324 FC1 viruses in 2005. Similarly, the A684T and A661T substitutions were identified in recent
325 FC1 viruses since 2011 and 2015, respectively. Positions 661 and 684 are known as markers
326 for mammalian adaptation in other influenza viruses [33, 64–67].

327 **PB1-F2.** PB1-F2 is an accessory protein (influenza viruses circulating in humans and other
328 mammalian species do not encode this polypeptide) that is usually 90 amino acids long and
329 displays proinflammatory properties [37]. In mice, amino acids L62, R75, R79, and L82 from
330 influenza A viruses were sufficient to generate an inflammatory response. Mutations at these
331 four positions are sufficient to attenuate the pro-inflammatory properties of the protein. It was
332 thus suggested that some PB1-F2 noninflammatory motifs (P62, H75, Q79, and S82) may
333 diminish the risk of secondary bacterial infection [37]. Moreover, it was experimentally
334 validated that the PB1-F2 proinflammatory motif increased morbidity in primary viral infection
335 and enhanced secondary bacterial infection in mice. Our study as well as [11] shows that the
336 A/equine/Beuvron-en-Auge/2/2009 strain displays a pro-inflammatory motif (L62, R75, and
337 R79) when compared to the A/equine/Paris/1/2018 virus with only L62 and R75. However,
338 another marked difference between these two PB1-F2 is their length. While that of

339 *A/equine/Beuvron-en-Auge/2/2009* is only 81 amino acids long, PB1-F2 encoded by
340 *A/equine/Paris/1/2018* is 9 amino acids longer with a sequence pattern alternating charged and
341 hydrophobic residues and a hydrophobic residue at position 82, a tryptophan. Full-length
342 versions of PB1-F2 (predominantly 87 or 90 amino acids) have been reported to specifically
343 translocate into mitochondria through their C-terminal region, which acts as a mitochondrial
344 targeting sequence and induces apoptosis [46, 53, 68, 69]. Our functional analyses (on cellular
345 mitochondria and synthetic membranes) reveal a different behavior of the 81- and 90-amino
346 acid-long PB1-F2. Membrane permeabilization was shown to be more efficient with the longer
347 than with the shorter (81 amino acid long) version of PB1-F2 on synthetic membranes. Both
348 forms were able to block the mitochondrial membrane potential when expressed in the cell
349 cytosol. We thus favor the hypothesis that both the length and the amino acid composition may
350 account for the contribution of PB1-F2 in virulence.

351 **Conclusion**

352 In conclusion, our study highlights the ongoing evolution of equine influenza viruses, with
353 subtle antigenic changes in hemagglutinin and unique genetic variations notably identified in
354 the *A/equine/Paris/1/2018* strain. Furthermore, this strain encodes a full-length accessory
355 protein, PB1-F2, resulting in higher permeabilization capacity when compared to shorter forms
356 and possibly contributing to its virulence. The use of advanced long-read sequencing
357 technologies appears to be imperative for monitoring subtle genetic variabilities of emerging
358 variants to identify key virulence markers in the ever-changing landscape of EIV.

359

360 **Materials and Methods**

361 **Cell Cultures**

362 A549 cells (human alveolar epithelial cells, American Type Culture Collection) and MDCK
363 cells (Madin-Darby Canine Kidney cells, ATCC) were cultured in minimal essential medium

364 (MEM) (Merck) containing 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg mL⁻¹
365 streptomycin, and 10% fetal bovine serum. Cells were maintained at 37°C in a 5% CO₂
366 incubator.

367 **Viruses**

368 Equine influenza viruses (EIV) H3N8 A/equine/Beuvron-en-Auge/2/2009,
369 A/equine/Paris/1/2018, and the vaccine strains A/equine/Richmond/1/2007 and
370 A/equine/South Africa/4/2003 were isolated from sick horses during respiratory disease
371 outbreaks. The nasopharyngeal swabs collected were placed in 5 ml of virus transport medium
372 containing minimum essential medium supplemented with 10% fetal bovine serum and 1% w/v
373 antibiotics (penicillin, streptomycin, and amphotericin).

374 EIV viruses were first amplified by passaging in 11-day-old embryonated chicken eggs (PA12
375 White Leghorn strain). Inocula were injected into the allantoic cavity (100 µl per egg). A second
376 virus amplification step was carried out in 25 cm² flasks of MDCK cell monolayers. When cell
377 lysis was observed, cultures were stopped, and RNA extraction was performed immediately.

378 **RNA extraction**

379 Extraction of EIV RNA from EIV-infected MDCK cells was carried out using TRIzol LS
380 Reagent (Life Technologies) and further purified using the RNeasy MinElute clean-up kit
381 (Qiagen) according to the manufacturer's recommendations. RNA integrity was assessed on an
382 Agilent 2100 Bioanalyzer using the RNA 6000 nano kit (Agilent, Santa Clara, CA) following
383 the manufacturer's instructions. We monitored RNA yield and purity with a NanoDrop ND-
384 2000c spectrophotometer.

385 **MinION long-read library preparation, sequencing and data analysis**

386 *cDNA synthesis*

387 Purified RNA was reverse transcribed using SuperScript III (Thermo Scientific) and primers
388 designed by Keller et coll. and complementary to the conserved 3' end of influenza A vRNA

389 [33]. We used primers RTA-U12 (5'-AGCAAAAGCAGG) expected to target the segments
390 PA, NP, M, NS and RTA-U12.4 (5'-AGCGAAAGCAGG) expected to target the segments
391 PB2, PB1, HA, NA, combined in a 2:3 molar ratio [33]. 500 ng of total RNA and 10 pmol of
392 specific primers (2:3 molar ratio RTA-U12, RTA-12.4) were denatured for 5 min at 65°C,
393 centrifuged, and stored on ice before adding the reaction mix, according to the manufacturer's
394 instructions. We incubated the RT reactions at 25°C for 10 min and then 50°C for 60 min. The
395 reaction was then stopped by heating at 70°C for 15 min. After cDNA synthesis, RNA was
396 degraded by incubation with 2 U of RNase H for 20 min at 37°C. The RNA hydrolysis reaction
397 was stopped by heating at 70°C for 10 min, and the cDNAs were stored at -20°C until use. We
398 evaluated the quantity and quality of cDNA on sixfold dilutions with the RNA 6000 Pico kit
399 (Agilent) on an Agilent 2100 Bioanalyzer.

400 cDNA amplification.

401 The eight influenza A genomic segments were amplified by PCR using the cDNA previously
402 produced. Platinum II Taq Hot start DNA-polymerase (Invitrogen) was used according to the
403 manufacturer's instructions, with primers set complementary to the 5' and 3' ends of each
404 influenza A genome segment (**Supp. Table 3**). Amplified DNA products were purified using
405 AMPure XP beads (Beckman Coulter Inc., Pasadena, CA, USA) at a ratio of 1.2:1 volume of
406 beads per sample, and DNA yield was monitored with a NanoDrop ND-2000c
407 spectrophotometer and a Qubit fluorimeter using a Qubit dsDNA BR kit (Invitrogen).

408 Nanopore sequencing and data analysis

409 For each of the four strains, the eight purified PCR products were pooled at an equimolar ratio
410 and used as input for library generation using the Ligation Sequencing Kit SQK-LSK109 and
411 the Native Barcoding Expansion 1-12 kit EXP-NBD104 according to the manufacturer's
412 instructions (Oxford Nanopore Technologies). The barcode-ligated DNA samples were pooled
413 at an equimolar ratio and used for final adapter ligation. We loaded 50 fmol of the purified

414 adapter-ligated DNA library onto a MinION Flow-cell (R9.4.1; FLO-MIN106D) and run it on
415 a MinION Mk1C device according to the manufacturer's instructions. Guppy (version 5.1.13)
416 was used for basecalling and demultiplexing. Nanofilt (version 2.8.0) was used to filter reads
417 based on their size and quality: size > 600 bp, Q>10. The filtered reads were mapped using
418 minimap2 (version 2.22) and *A/equine/Ohio/113461-1/2005* as the reference genome
419 (GenBank accession numbers: CY067323, CY067324, CY067325, CY067326, CY067327,
420 CY067328, CY067329, CY067330). SAMtools (version 1.14) was used to convert the data into
421 bam and medaka (version 1.4.4) for variant calling. Finally, the Integrative Genomics viewer
422 desktop application (IGV, version 2.16.2) was used for visualization.

423 The newly sequenced viral genomes have been deposited in the European Nucleotide Archive
424 under project accession number X, available at www.ebi.ac.uk/ena.

425 **Sequence multialignment and phylogenetic trees**

426 A multiple alignment of all nucleotide sequences of the eight genes of equine influenza of type
427 A H3N8 was obtained using the Muscle algorithm and the maximum likelihood method and
428 Hasegawa-Kishino-Yano model [70]. The tree with the highest likelihood is shown. The
429 percentage of replicate trees in which the associated taxa clustered together in the bootstrap test
430 1000 replicates [71] are shown next to the branches. Initial tree(s) for the heuristic search were
431 obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of
432 pairwise distances estimated using the maximum composite likelihood (MCL) approach and
433 then selecting the topology with superior log likelihood value. A discrete Gamma distribution
434 was used to model evolutionary rate differences among sites (5 categories (+G, parameter)).
435 The codon positions included were 1st+2nd+3rd+Noncoding. Evolutionary analyses were
436 conducted in MEGA11 [72, 73]. All accession numbers are listed in **Supp. Table 1**.

437 The amino acid sequences of viral proteins (PB2, PB1, PB1-F2, PA, PA-X, HA, NP, NA, M1,
438 M2, NS1, and NEP) from recent EIV isolated in France were aligned with the strain

439 A/equine/Ohio/1/2005 used as a reference for consensus sequence construction using Clustal
440 Omega from EMBL-EBI [74] and Unipro UGENE [75].

441 **Plasmids**

442 Codon-optimized open reading frames encoding HA-tagged versions of PB1-F2 of viral strains
443 A/equine/Ohio/1/2003 and A/equine/Paris/1/2018 were cloned in the eukaryotic expression
444 vector pCAGGS at the Not I and Bgl II restriction sites. Codon-optimized open reading frames
445 encoding His-tagged versions of PB1-F2 of A/equine/Ohio/1/2003 and A/equine/Paris/1/2018
446 were cloned in the bacterial expression vector pET-28a+ at the Nde I and Xho I restriction sites.

447 **Immunohistochemistry - Confocal Microscopy**

448 A549 cells were seeded at 0.5×10^6 cells per well on 18 mm diameter glass lamellas and
449 incubated for 24 h at 37°C and 5% CO₂. Cells at 80~90% confluence were transfected with
450 200 ng of pCAGGS derivatives using Lipofectamine® 2000 (11668027, Thermo Fisher
451 Scientific) following the manufacturer's instructions. Forty hours after transfection,
452 MitoTracker CMX Ros (M7512, Thermo Fisher Scientific) was added to the cell culture at a
453 final concentration of 500 nM for 30 min. Next, after cell culture medium removal, the cells
454 were fixed using 4% paraformaldehyde for 30 min at room temperature (RT). Cell monolayers
455 were washed in phosphate saline buffer (PBS) and PBS completed with 0.1% Triton X-100
456 (PBS-Tx) and with 1% w/v bovine serum albumin (BSA) for 1 h at RT. The cells were then
457 incubated with a rabbit anti-HA-tag antibody (H6908, Sigma-Aldrich) in PBS-Tx
458 supplemented with 0.2% BSA. After three washes in PBS-Tx, an anti-rabbit immunoglobulin
459 goat antibody labeled with Alexa Fluor 488 (A11008, Invitrogen, OR, USA) in PBS-Tx
460 completed with 0.2% BSA was added for 2 h at RT. Nuclei were marked with Hoechst diluted
461 to 1/100 in PBS 1x for 5 min at RT. Subcellular localization images were taken using a Zeiss
462 LSM 700 confocal 187 microscope with a x63 objective.

463 **PB1-F2 production in *E. coli* and purification**

464 BL-21 Rosetta cells (Stratagene) were transformed with the resulting plasmids and cultured to
465 an optical density (OD) of 0.8 before overnight incubation at 28°C in 1 mM isopropyl 1-thio-
466 β-D-galactopyranoside (IPTG) under agitation. Next, bacteria were pelleted and resuspended
467 in 50 mM Tris (pH 7.4), 10 mM EDTA, and 0.1% Triton X-100 buffer and incubated at 37°C
468 for 30 min. The suspension was sonicated and centrifuged at 10,000 × g for 30 min at 4°C.
469 Pellets were resuspended in solubilization buffer (20 mM Tris (pH 7.4), 0.5 M NaCl, 5 mM
470 imidazole, and 8 M urea) and centrifuged at 10,000 × g for 30 min at 4°C. Supernatants were
471 sonicated and filtrated using 0.8 μm filters (SLAAR33S, MilliporeSigma™ Millex™) before
472 loading on a Histrap FF IMAC column (17531901, Cytiva) using the AKTA Purifier 100 FPLC
473 chromatographic system (GE Healthcare). Fractions containing PB1-F2 were pooled and
474 subjected to size exclusion chromatography on a Sepharose S200 column equilibrated with
475 solubilization buffer. Next, urea was removed from the S200 PB1-F2-containing fractions on a
476 53 mL HiPrep™ 26/10 Sephadex G-25 resin column (GE17-5087-01, Sigma–Aldrich)
477 equilibrated with 5 mM ammonium acetate buffer, pH 5. Fractions containing PB1-F2 were
478 lyophilized and stored at –20°C. Prior to their use, lyophilized PB1-F2 powder was dissolved
479 in 5 mM sodium acetate buffer (pH 5). Protein concentration was estimated by measuring OD
480 at 280 nm and using the extinction coefficients of 23490 M⁻¹cm⁻¹ for the Ohio protein and
481 37470 M⁻¹cm⁻¹ for its Paris homolog.

482 **Lipid vesicle preparation**

483 (16:0-18:1) 1-Palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) (840034), 1-
484 palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (PG) (840457), and (18:1)
485 cardiolipin 1',3'-bis[1,2-dioleoyl-sn-glycero-3-phospho]-glycerol (DOCL) (840044) were
486 purchased from Avanti Polar Lipids (Alabaster, AL, USA). (16:0-18:1) 1-Palmitoyl-2-oleoyl-
487 glycero-3-phosphocholine (POPC) (37-1618-9), (16:0-18:1) 1-palmitoyl-2-oleoyl-sn-glycero-
488 3-phosphoethanolamine (POPE) (37-1828-7), and soybean L-α-phosphatidylinositol (PI) (37-

489 0130-7) were purchased from Larodan (France). ANTS (FP-46574B, 8-aminonaphthalene-1,3,6
490 trisulfonic acid) and DPX (FP-47017A, p-xylene-bis-pyridinium bromide) were purchased
491 from Interchim (Montluçon, France). Sodium acetate buffers and phosphate buffers were of
492 analytical grade. Reagents for SDS-PAGE electrophoresis were obtained from Invitrogen
493 (France).

494 Lipids POPC, POPE, POPS, PI, and DOCL were used at a molar ratio of 5.5:2.5:1.5:1:0.5 to
495 mimic mitochondria outer membranes (OMM). The mix of lipids with 20 mM ANTS
496 (fluorophore probe) and 60 mM DPX (quencher) in a final concentration of 10 mM sodium
497 acetate (pH 5) was sonicated using a sonicator tip to obtain an emulsion. Reversed-phase
498 evaporation was carried out using a Heidolph Laborota 4003 apparatus to obtain large
499 unilamellar vesicles (LUVs). LUV preparations were extruded three times through a Swinny
500 filter (XX3001200, Millipore) using polycarbonate filters with pore size diameters of 1.2 μm ,
501 0.4 μm and 0.2 μm (Merck Millipore, Darmstadt, Germany). Unencapsulated ANTS and DPX
502 were removed by gel filtration through a 5 mL HiTrap Desalting Sephadex G-25 resin column
503 (GE Healthcare Life Sciences). To ensure the correct size and obtain LUVs, dynamic light
504 scattering (DLS) measurements were performed on a Nano series Zetasizer (Malvern
505 Instruments, Paris, France).

506 **Lipid vesicle permeabilization assay**

507 For permeabilization assays, LUVs were incubated at 0.4 mM lipid concentration in 10 mM
508 sodium acetate (pH 5) at 25°C in a black p96-well plaque (Greiner), and fluorescence titrations
509 were performed with an FP-8200 Jasco spectrofluorometer equipped with a Peltier-
510 thermostated ETC-272T (25°C). The excitation wavelength was set at 360 nm, and the emission
511 of ANTS was measured between 500-600 nm at a bandwidth of 5 nm to ensure that the signal
512 perceived was indeed permeabilization and not unspecific diffraction. The intensity was
513 measured before and after the addition of PB1-F2 at final concentrations of 1 μM , 500 nM, 250

514 nM, 100 nM, and 50 nM. The maximum intensity of permeabilization, corresponding to the
515 maximum intensity of ANTS fluorescence, was measured after the addition of 0.1% (v/v) Triton
516 X-100. The experiment was carried out 4 times in triplicate. Statistical analysis was carried out
517 with REML $F(1,99) = 55.01$, $P < 0.0001$ and Šídák's multiple comparison (1 μ M P value =
518 0.0021; 500 nM P value = 0.0011; 250 nM P value=0.0003) on Prism v9.

519

520 **Figure legends**

521 **Figure 1: Schematic workflow implemented for long-read sequencing of equine influenza**

522 **virus.** The four equine influenza viruses A/equine/Beuvron-en-Auge/2/2009,
523 A/equine/Paris/1/2018, and OIE recommended vaccine strains A/equine/South Africa/4/2003
524 and A/equine/Richmond/1/2007 were analyzed. (A) After viral amplification in the MDCK cell
525 line and RNA extraction, the eight genomic segments were individually amplified by RT-PCR.
526 Amplified DNA products were controlled by capillary electrophoresis. (B) For each strain, the
527 eight amplicons were pooled with equimolar ratios, and sequencing libraries were prepared and
528 loaded on a flow cell. (C) The bioinformatics workflow used from raw data to consensus
529 sequence construction. The reference strain is A/equine/Ohio/2005 (GenBank accession
530 numbers: CY067323, CY067324, CY067325, CY067326, CY067327, CY067328, CY067329,
531 CY067330).

532

533 **Figure 2: Nucleotidic variation patterns.** This graphic extracted from Integrative Genomics

534 Viewer [76] depicts variants as vertical bars along the x-axis for the different sequences shown
535 on the y-axis. The four consensus genomic sequences of A/equine/Paris/1/2018 (Paris),
536 A/equine/Richmond/1/2007 (Richmond), A/equine/South Africa/4/2003 (South Africa) and
537 A/equine/Beuvron-en-Auge/2/2009 (Beuvron) are aligned to the reference

538 (A/equine/Ohio/113461-1/2005 sequences) to visualize the variation patterns across the strains.

539 The scale is indicated for each segment.

540

541 **Figure 3: Phylogenetic analysis of the HA (A) and NA (B) nucleotide sequences for 27 EIV**

542 **strains.** The analysis includes representative strains of the main lineages, sublineages, and

543 vaccine strains (*). Phylogenetic trees were created using the maximum likelihood method and

544 Hasegawa-Kishino-Yano model with 1000 bootstraps.

545

546 **Figure 4: Phylogenetic analysis of the nucleotide sequences encoding PB2 (A), PB1 (B),**

547 **PA (C), NP (D), M (E) and NS (F).** The analysis includes representative strains of the main

548 lineages, sublineages, and vaccine strains. Phylogenetic trees were created using the maximum

549 likelihood method and Hasegawa-Kishino-Yano model with 1000 bootstraps.

550

551 **Figure 5: HA antigenic sites.** (A) Amino acid alignments of the five antigenic sites A to E

552 with HA sequences determined for French strains and other fully sequenced viral strains and

553 compared with A/equine/Miami/1/1963. The antigenic sites defined for the human H3 influenza

554 virus were used as a reference [28, 29, 31]. (B) Lateral and top views of the 3D structure of H3

555 hemagglutinin (PDB accession number: 4UO0) and location of its antigenic sites. While the

556 HA2 domain (in pink and magenta) constitutes the stem, HA1 domains form the head of the

557 HA bearing the antigenic sites. Antigenic sites are colored in cyan (site A), orange (site B),

558 green (site C), red (site D), and yellow (site E). (C) Location of HA amino acid substitutions

559 between the FC1 strains A/equine/Ohio/1/2003 and A/equine/Paris/1/2018. Amino acid

560 changes are colored according to their positions in the corresponding antigenic sites (as in (B))

561 or in blue. (D) Location of HA amino acid substitutions between the FC2 strains

562 A/equine/Richmond/1/2007 and A/equine/Saone-et-Loire/1/2015. Color patterning as in (C).

563

564 **Figure 6: Positions of the amino acid substitutions on the surface of N8 between the FC1**
565 **vaccine strain A/equine/Ohio/1/2003 and the A/equine/Paris/1/2018 strain.** Only the head
566 of NA is represented. Amino acid changes are colored blue, and catalytic residues are colored
567 yellow. The 3D structure template is the PDB accession number 2HT5.

568

569 **Figure 7: Amino acid sequence comparison between the French strains and the OIE-**
570 **recommended vaccine strains.** Amino acid identity to A/equine/Ohio/1/2003 is represented
571 as a dot.

572

573 **Figure 8: PB1-F2 amino acid sequence comparison.** The analysis includes the
574 A/equine/Beuvron-en-Auge/2/2009 and A/equine/Paris/1/2018 strains as well as representative
575 strains. Amino acid identity to A/equine/Miami/1/1963 is represented as a dot.

576

577 **Figure 9: Comparison of biological properties of the virulence factor PB1-F2 of**
578 **A/equine/Ohio/1/2003 and A/equine/Paris/1/2018.**

579 **(A)** Disruption of mitochondrial membrane potential ($\Delta\Psi_m$) in A549 cells expressing HA-
580 tagged PB1-F2 variants from A/equine/Paris/1/2018 (HA-PB1-F2_{PARIS2018}) and
581 A/equine/Ohio/1/2003 (HA-PB1-F2_{OHIO2003}) viruses. Cells were fixed 48 h post transfection
582 and processed for indirect immunofluorescence staining with an anti-HA-tag rat antibody and
583 an anti-rat secondary antibody coupled with Alexa Fluor 488 (green). Mitochondria were
584 revealed using the $\Delta\Psi_m$ -sensitive mitochondrial dye MitoTracker CMX Ros (magenta), and
585 nuclei were revealed with Hoechst (blue). Scale bars, 10 μm . **(B)** Membrane permeabilization
586 assay using recombinant forms of PB1-F2 encoded by A/equine/Paris/1/2018 (PB1-F2_{PARIS2018})
587 and A/equine/Ohio/1/2003 (PB1-F2_{OHIO2003}) viruses. LUVs mimicking mitochondrial outer-

588 membrane composition containing the fluorophore probe (ANTS) and quencher (DPX) were
589 incubated with serial dilutions of PB1-F2 forms. The experiment was carried out 4 times in
590 triplicate. Statistical analysis was carried out with REML $F(1,99) = 55.01$, $P < 0.0001$, and
591 Šídák's multiple comparison. P values are indicated in the figure.

592

593 **Additional file legends**

594 **Supp Fig. 1.ppt**

595 **Unique and shared nucleotide variations.**

596 This graphic represents each identified variant as a dot along the x-axis, according to the
597 number of strains that contained it along the y-axis. On the right, the bar plot represents the
598 total number of variants that are either unique to a strain (N=1) or shared between two to four
599 of the analyzed strains.

600

601 **Supp Fig. 2.ppt**

602 **Number of nucleotide substitutions.**

603 The reference sequence used was A/equine/Ohio/113461-1/2005. The number of substitutions
604 per segment and by strain is shown in black and strain-specific in gray.

605

606 **Supp. Fig 3.ppt**

607 **Multiple alignment of HA amino acid sequences for selected strains since 1963.**

608 Antigenic sites are indicated in blue outlined boxes. Amino acid identity is represented with a
609 dot. Absent amino acids are represented with a line.

610 Blue letters (A-E) indicate the antigenic sites. The 130-loop, 190-helix, and 220-loop involved
611 in the receptor-binding site are indicated in orange outlined boxes.

612

613 **Supp. Fig 4.ppt**

614 **Multiple alignment of NA amino acid sequences for selected strains since 1963.** Amino
615 acid identity is represented with a dot. Absent amino acids are represented with a line.

616

617 **Supp. Table 1.ppt**

618 **Accession numbers of all selected sequences used for phylogenetic analyses.**

619

620 **Supp. Table 2.ppt**

621 **Substitutions found in HA and NA.**

622 Comparison to A/equine/Ohio/1/2003 for 2009 and 2018 French strains, strain used for
623 MinION consensus sequence, OIE recommended vaccine strains A/equine/South
624 Africa/4/2003 (Fc1) and A/equine/Richmond/1/2007 (Fc2). Numbering according to mature
625 HA. Lines represent identity to A/equine/Ohio/1/2003.

626

627 **Supp Table 3.ppt**

628 **Primer sequences for viral genomic segment amplification.**

629

630 **Declarations**

631 **Availability of data and materials**

632 The datasets used and/or analyzed during the current study are available from the corresponding
633 author upon reasonable request.

634 **Competing interests**

635 The authors declare that they have no competing interests.

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661

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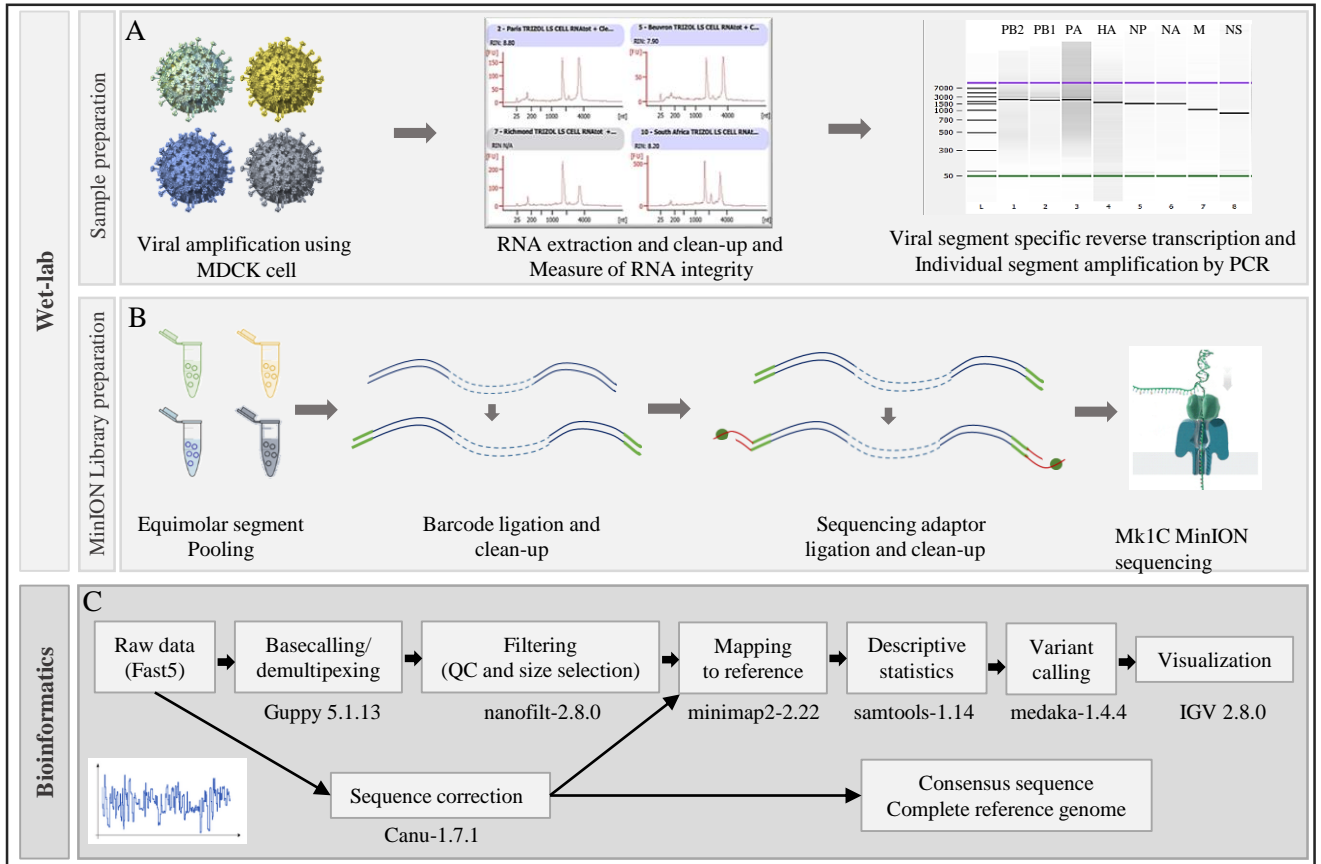


Fig 1 KLEIJ et al., 2023

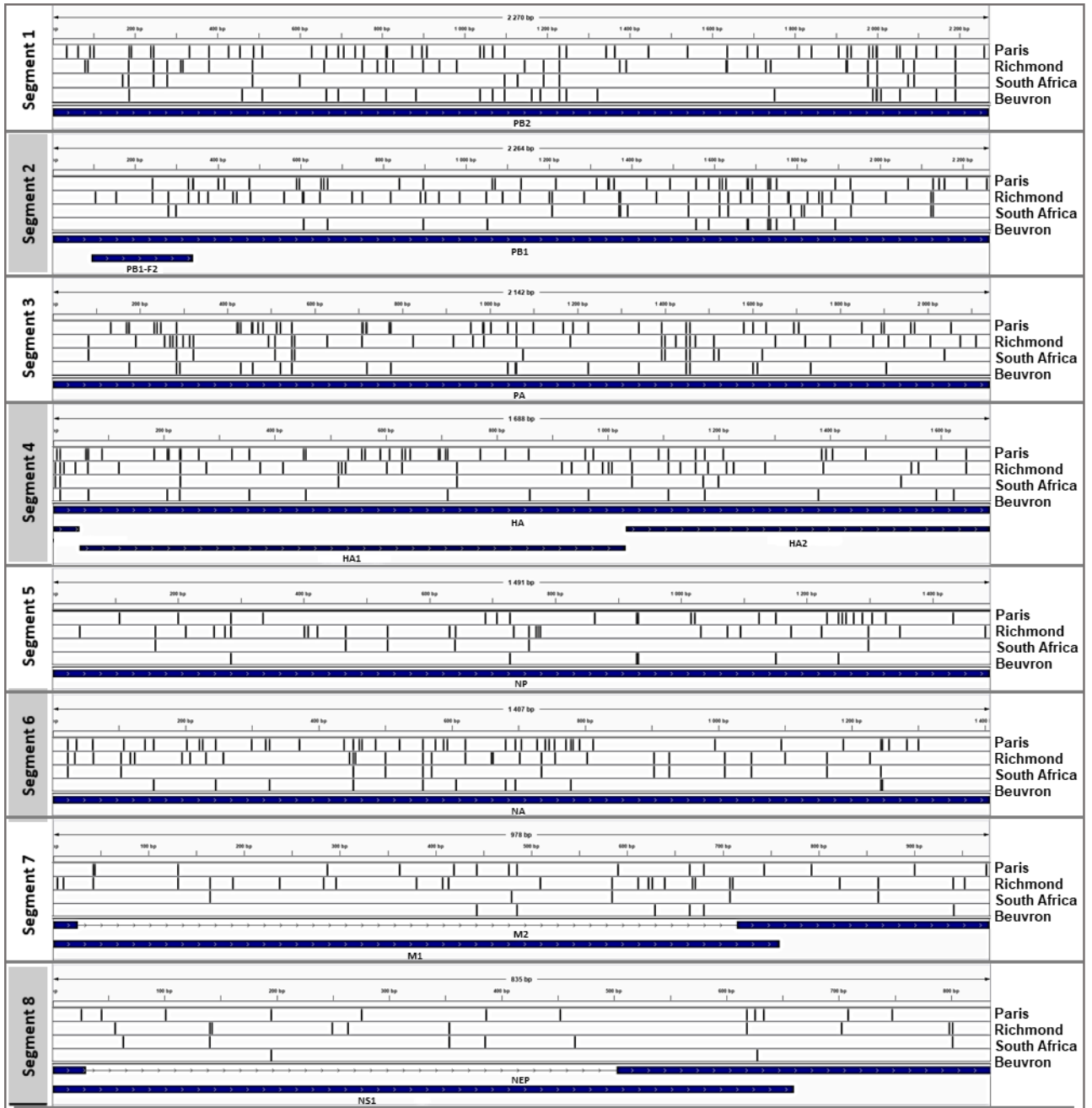
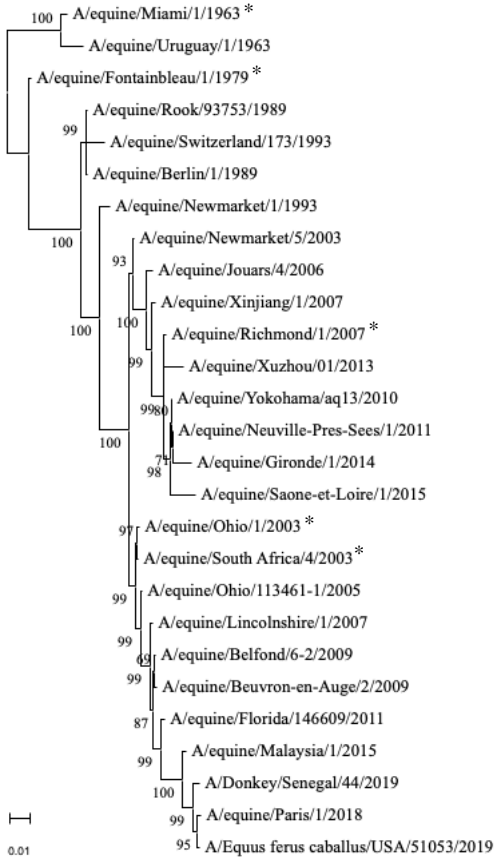


Fig 2 KLEIJ et al., 2023

A. HA



B. NA

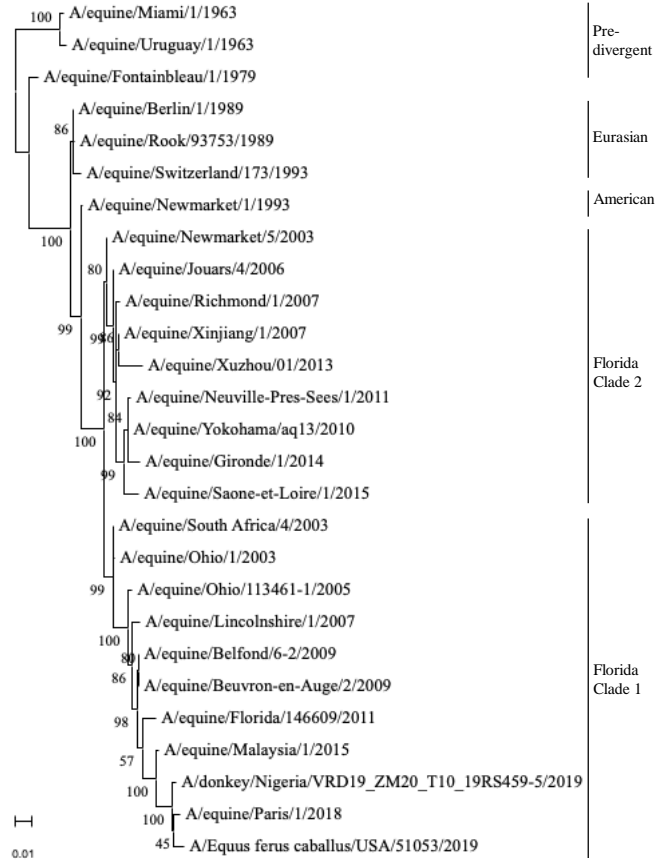
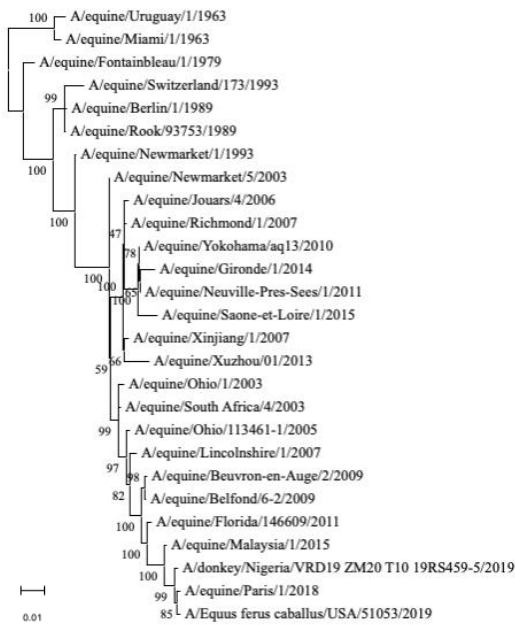
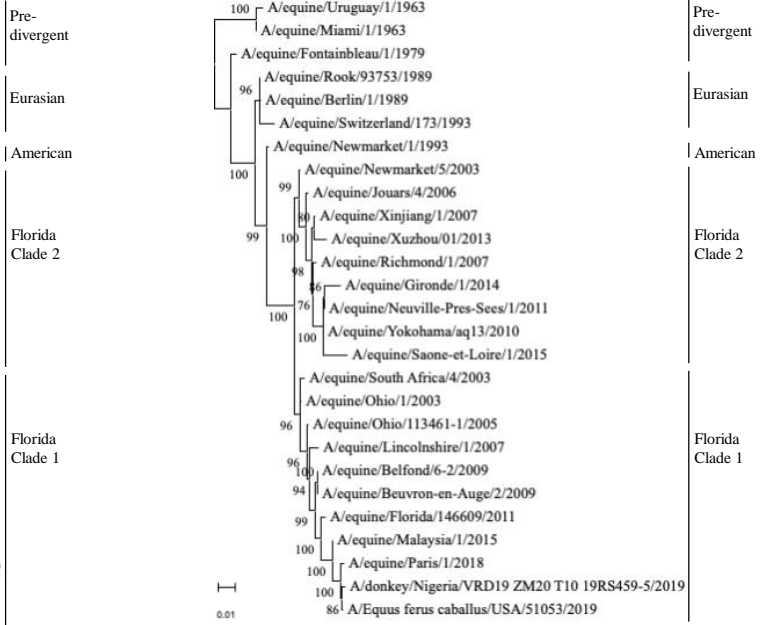
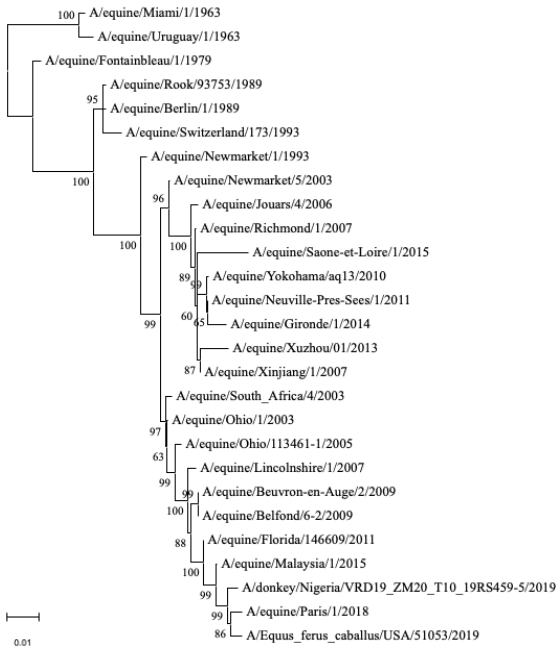
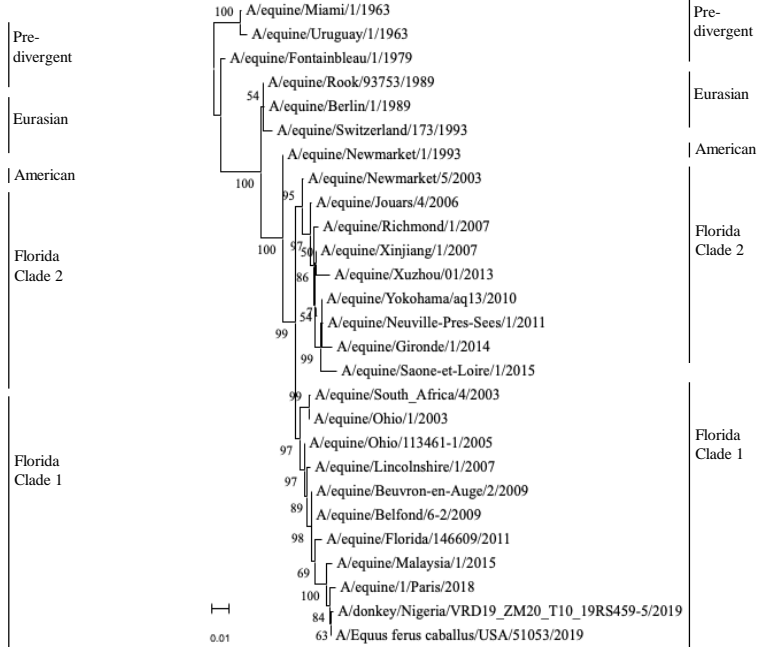
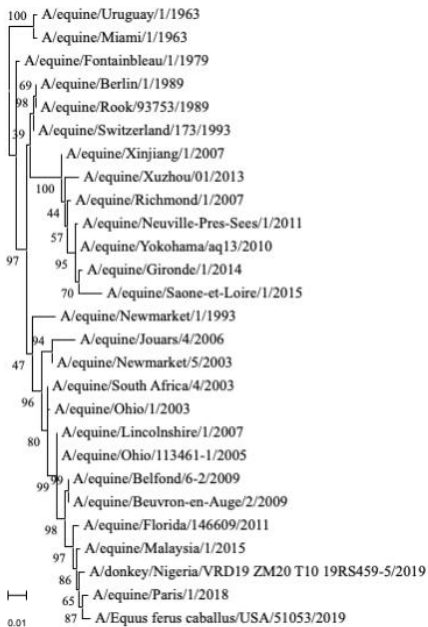
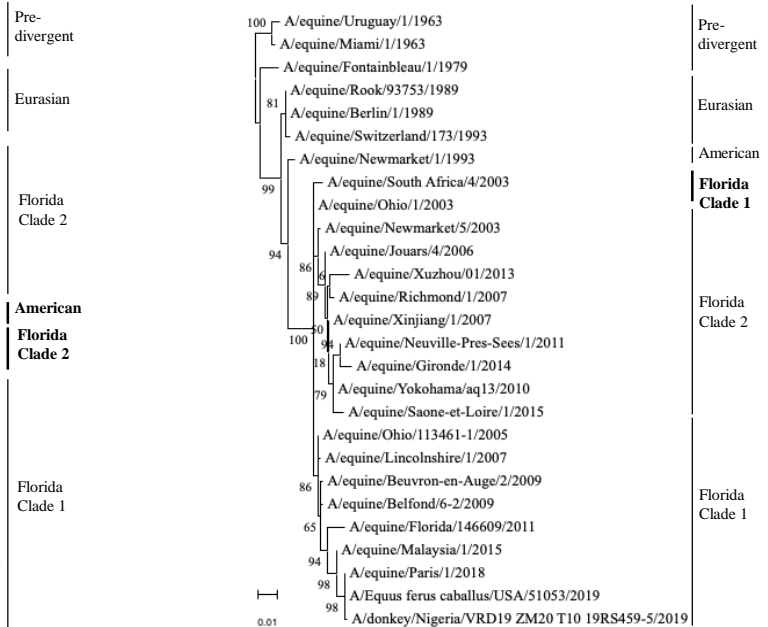


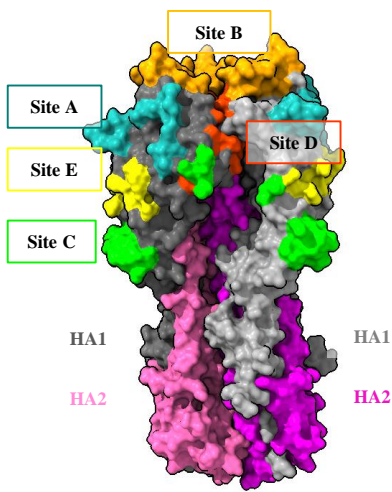
Fig 3 KLEIJ et al., 2023

A. PB2**B. PB1****C. PA****D. NP****E. M****F. NS**

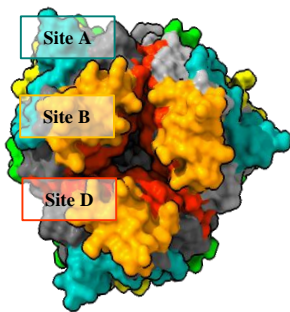
A.

Clade	H3 antigenic sites	Site A		Site B		Site C		Site D		Site E		
		121-128	132-146	155-163	186-199	48-55	273-278	170-174	201-220	241-248	62-63	78-83
pre-divergent	A/equine/Miami/1/1963	M A E G P T W T	Q N G G S S A C R R G S A D S	T K S E S S Y P T	S T N N E Q T K L Y V Q A S	T G R I C I N N P	P I D T C V	N N D N F	R V T V S T K R S Q Q T I P N I G S R	D V L M I N S N	R N	V F Q Y E N
	A/equine/Uruguay/1/1963	S		G N	I							G
	A/equine/Fontainebleau/1/1979	T	G	G N	E L		N			I		
Eurasian	A/equine/Rook/93753/1989	T	R G K	G N	I S K	I E	S	K	E V	I T		
	A/equine/Berlin/1/1989	T	G	G N	I S K	I E	S	K	E V	I		
	A/equine/Switzerland/173/1993	T	R G K	N G N	I S K	T E	S L	K	E V R	I		D
American	A/equine/Newmarket/1/1993	T	R G K	G N	S O Q	E I E	S	I	K			I
Florida Clade 2	A/equine/Newmarket/5/2003	T	R G K	G N	S O	I E	S	I	K			I
	A/equine/Jouars/4/2006	T	R G K	G N	S O	I E	S	I	K	M		
	A/equine/Xinjiang/1/2007	T	R G K	E G N	S O	I E	S	I	K			
	A/equine/Richmond/1/2007	T	R G K	G N	S O	I E	S	I	K			
	A/equine/Yokohama/aq13/2010	T	R G K	G N	S O	I E	S	I	K			
	A/equine/Neuville-Pres-Sees/1/2011	T	R G K	G N	S O	I E	S	I	K			
	A/equine/Xuzhou/01/2013	T	R G K	G N	S O	I G	S	V	K			
	A/equine/Gironde/1/2014	T	R G K	V	G N	S O	I E	S	I	K		
	A/equine/Saone-et-Loire/1/2015	T	R G K	T	G N	S O	K I R E	S	I	K		
	Florida Clade 1	A/equine/Ohio/1/2003	T	R G K	G	S O	I E	S	I	K		
A/equine/South Africa/4/2003		T	R G K	G	S O	I E	S	I	K			A
A/equine/Ohio/113461-1/2005		T	R G K	G	S O	I E	S	I	K			A
A/equine/Lincolnshire/1/2007		T	R G S K	G	S O	I E	S	I	K			K A
A/equine/Belfond/6-2/2009		T	R G S K	G	S O	I E	S	I	K			K A
A/equine/Beuvron-en-Auge/2/2009		T	R G S K	G	S O	I E	S	I	K			K A
A/equine/Florida/146609/2011		T	R G S K	G	S O	I E	S	I	K			K A
A/equine/Malaysia/1/2015		T	R G S K	G	S T O	I E	S	I	K			K A
A/equine/Paris/1/2018		T	R G S K	G	I S T O	I E	S	I	K			K D A
A/Equus ferus caballus/USA/51053/2019		T	R G S K	G	S T O	I E	S	I	K			K D A
A/donkey/Senegal/44/2019	T	R G S K	G	S T O	I E	S	I	K			K D A	

B.

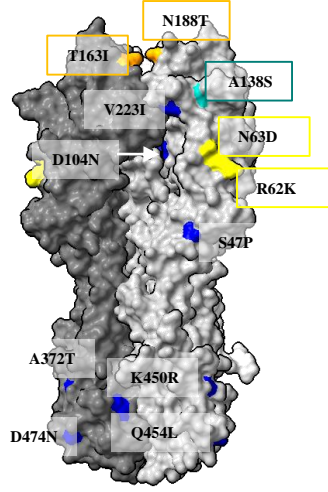


Lateral surface view

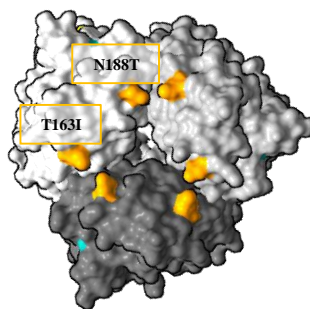


Top surface view

C.

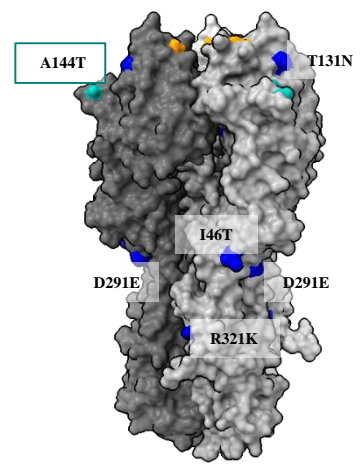


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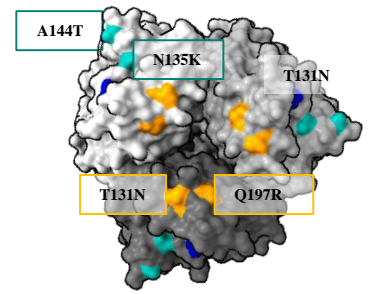


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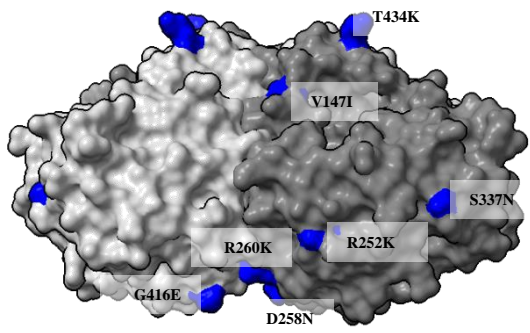
D.



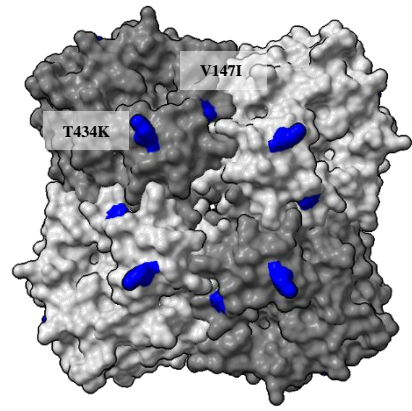
Lateral surface view



Top surface view



Lateral surface view



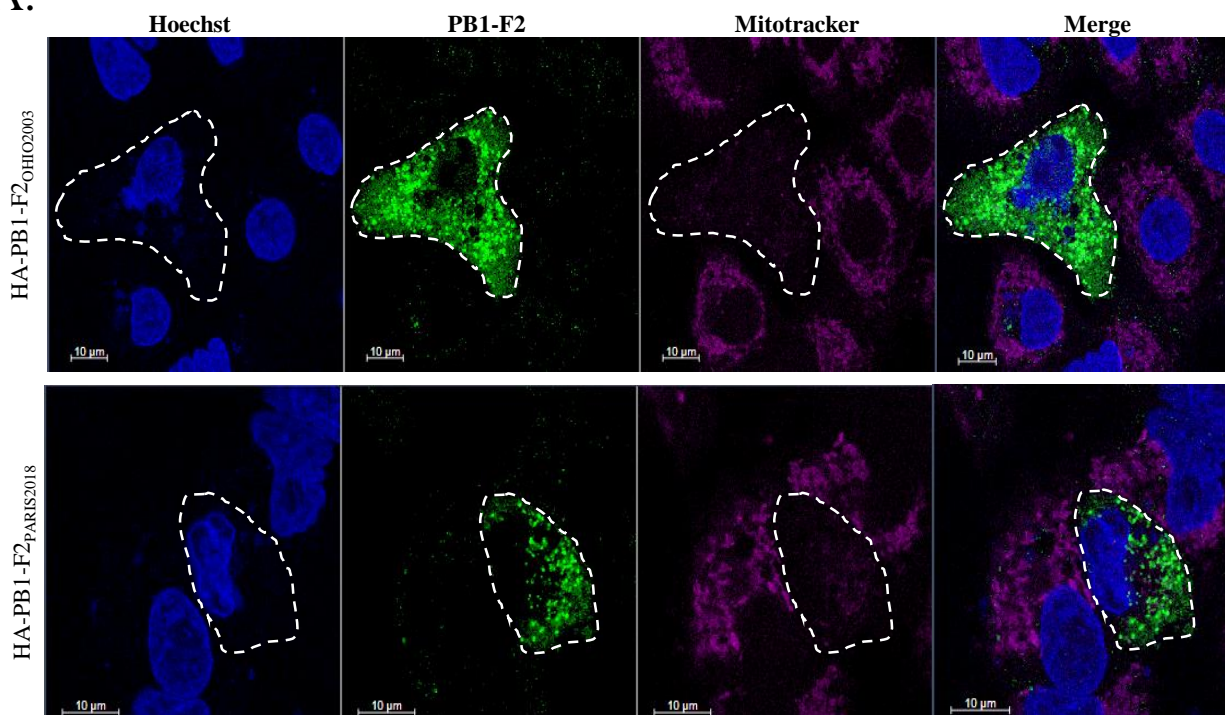
Top surface view

	PB2														PB1																
Amino acid position	63	65	105	251	295	377	395	398	411	660	661	667	684	686	699	731	754	94	114	119	200	203	329	377	578	584	618	621	644	715	754
A/equine/Ohio/1/2003	I	E	T	R	V	A	A	I	I	K	A	V	A	V	K	V	I	F	I	V	V	R	Q	D	K	R	E	K	V	V	R
A/equine/South Africa/4/2003	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
A/equine/Ohio/113461-1/2005	-	-	-	-	-	-	-	V	V	R	-	-	-	-	-	I	-	L	-	-	-	-	-	I	-	-	R	-	-	-	
A/equine/Richmond/2007	-	-	A	K	-	-	-	V	-	-	-	-	-	-	-	-	-	L	-	M	-	-	R	E	-	-	D	-	-	-	
A/equine/Beuvron-en-Auge/2/2009	V	-	-	-	I	-	V	V	-	-	-	I	-	I	-	-	-	L	-	-	-	K	-	-	-	Q	-	R	-	-	
A/equine/Paris/1/2018	V	K	-	-	-	-	-	V	-	-	T	I	T	I	R	-	V	L	V	-	I	-	-	-	-	Q	-	R	I	A	G

	PA														NP											
Amino acid position	59	64	86	98	210	237	259	321	335	348	354	367	409	465	476	505	538	626	636	136	214	257	359	430	450	
A/equine/Ohio/1/2003	E	E	M	T	T	E	P	S	L	L	T	K	S	I	A	V	E	K	V	M	R	I	T	T	N	
A/equine/South Africa/4/2003	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	-	-	-	-	K	-	-	-	-	
A/equine/Ohio/113461-1/2005	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	
A/equine/Richmond/2007	-	D	I	-	M	K	-	N	-	-	I	-	-	-	T	I	-	R	-	I	-	T	A	-	S	
A/equine/Beuvron-en-Auge/2/2009	-	-	-	A	-	-	S	-	-	I	I	-	N	V	-	-	K	-	I	-	-	-	-	-	-	
A/equine/Paris/1/2018	K	-	-	-	-	K	S	-	I	I	I	R	N	-	-	-	-	-	-	-	-	-	-	-	I	-

	M1			M2			NSI										NEP					
Amino acid position	15	80	95	208	214	248	19	59	85	87	89	22	48	66	84	129	156	207	209	210	212	52
A/equine/Ohio/1/2003	V	V	R	R	Q	M	C	L	D	E	G	F	S	E	V	I	V	H	N	G	P	M
A/equine/South Africa/4/2003	-	-	-	-	-	-	-	-	-	D	-	V	-	-	-	T	I	-	-	-	-	-
A/equine/Ohio/113461-1/2005	-	-	-	-	-	-	-	M	-	D	-	-	-	-	-	-	-	-	-	-	-	-
A/equine/Richmond/2007	I	I	K	K	E	-	-	-	S	D	S	-	I	-	I	-	-	Y	-	-	-	-
A/equine/Beuvron-en-Auge/2/2009	-	-	-	-	-	-	-	M	G	D	-	-	-	K	-	-	-	-	-	W	-	I
A/equine/Paris/1/2018	I	-	-	-	-	I	Y	M	-	D	-	-	-	K	-	-	-	N	I	-	S	L

A.



B.

