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1 Isolation and characterisation of equine influenza viruses (H3N8) from Europe and North
2 America from 2008 to 2009

3

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27 Abstract

28 Like other influenza A viruses, equine influenza virus undergoes antigenic drift. It is
29 therefore essential that surveillance is carried out to ensure that recommended strains for
30 inclusion in vaccines are kept up to date. Here we report antigenic and genetic
31 characterisation carried out on equine influenza virus strains isolated in North America and
32 Europe over a two year period from 2008 to 2009. Nasopharyngeal swabs were taken from
33 equines showing acute clinical signs and submitted to diagnostic laboratories for testing and
34 virus isolation in eggs. The sequence of the HA1 portion of the viral haemagglutinin was
35 determined for each strain. Where possible, sequence was determined directly from swab
36 material as well as from virus isolated in eggs. In Europe, 20 viruses were isolated from 15
37 sporadic outbreaks and 5 viruses were isolated from North America. All of the European and
38 North American viruses were characterised as members of the Florida sublineage, with
39 similarity to A/eq/Lincolnshire/1/07 (Clade 1) or A/eq/Richmond/1/07 (Clade 2). Antigenic
40 characterisation by haemagglutination inhibition assay indicated that the two clades could be
41 readily distinguished and there were also at least seven amino acid differences between them.
42 The selection of vaccine strains for 2010 by the Expert Surveillance Panel have taken these
43 differences into account and it is now recommended that representatives of both Florida
44 Clade 1 and Clade 2 are included in vaccines.

45

46 Keywords: Equine Influenza virus, H3N8, surveillance, vaccine strain selection

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48

49 1. Introduction

50 Equine influenza virus (EIV) of the family *Orthomyxoviridae* is a major cause of
51 respiratory disease in the horse. The two subtypes H7N7 and H3N8 have been isolated from
52 horses, and from 1963 to the late 1970s both subtypes co-circulated (Sovinova et al., 1958;
53 Waddell et al., 1963). The equine H7N7 viruses have not been isolated since 1979, however
54 the H3N8 viruses have continued to circulate to this day (Bryant et al., 2009b; Damiani et al.,
55 2008; Rozek et al., 2009; Webster, 1993). Since the initial cross species transmission of
56 H3N8 into horses that caused the 1963 epidemic in the Americas, there have been numerous
57 large outbreaks of disease in naïve and vaccinated horse populations that have had severe
58 financial implications for the horse industry (Callinan, 2008; Daly et al., 2004; Ito et al.,
59 2008; Newton et al., 2006; Virmani et al., 2010).

60 EIV diverged into the Eurasian and American lineages in the late 1980s, and the
61 American lineage has diverged further into the Kentucky, South American and Florida
62 sublineages clades 1 and 2 (Daly et al., 1996; Lai et al., 2001; OIE, 2008). Data from 2006 to
63 2007 showed the majority of EIVs circulating in Europe belonged to the Florida sublineage
64 clade 2 while those circulating in North America belonged to the Florida sublineage clade 1
65 (Bryant et al., 2009b; Gagnon et al., 2007; Rozek et al., 2009). Viruses responsible for the
66 large outbreaks in Japan and Australia were classified as Florida sublineage clade 1
67 (Callinan, 2008; Yamanaka et al., 2008). Viruses from Mongolia in 2008 and the 2009
68 outbreak in India were both classified as Florida sublineage clade 2 (Virmani et al., 2010).

69 Vaccination is the most effective method of prophylaxis against equine influenza.
70 Vaccines provide protection by inducing antibody to the viral surface glycoproteins, in
71 particular the haemagglutinin (HA), with some vaccines providing protection through

72 stimulation of cell mediated immunity (Paillot et al., 2006). Whether protection induced by
73 one virus strain is effective against another is dependent on the antigenic differences between
74 them (Haaheim and Schild, 1979; Yates and Mumford, 2000). Active surveillance of EIV is
75 undertaken at the Animal Health Trust in collaboration with international partners, in order to
76 monitor the antigenic variation of the surface haemagglutinin and other viral proteins from
77 circulating virus strains. The purpose of this activity is to provide data for helping the
78 selection of appropriate vaccine strains by the World Organisation for Animal Health (OIE).
79 These data are reviewed on an annual basis and, if appropriate, the recommendations are
80 changed to represent significant differences that have occurred to the viruses in circulation in
81 the field. Based on 2008 surveillance data, the 2009 OIE vaccine strain recommendation was
82 for the continued inclusion of an A/eq/South Africa/4/03-like virus representing the
83 American lineage, a recommendation first made in 2004. This virus was later classified as a
84 Florida sublineage clade 1 virus. The OIE no longer recommended the need for a Eurasian
85 strain such as A/eq/Newmarket/2/93 because of the lack of isolates from this lineage (OIE,
86 2009). This report summarises the EIV surveillance data collected at the Animal Health Trust
87 from the UK, Germany and the USA from 2008 to 2009 that has contributed to the expert
88 surveillance panel vaccine recommendations for 2010.

89

90 2. Materials and methods

91 2.1 NP-ELISA, Directigen Flu A, Binax Now Flu A and Optical ImmunoAssay

92 Nasopharyngeal swabs (Newton et al., 1997) were taken from horses in the UK
93 showing signs of acute respiratory disease. Swabs were placed in sterile tubes containing 5
94 mL virus transport medium (VTM) consisting of PBS, 200 U/mL streptomycin, 150 U/mL

95 penicillin, 5µg/mL fungizone (Gibco) and 600 µg/mL tryptone phosphate broth and chilled
96 on ice immediately after collection. An in-house nucleoprotein enzyme-linked
97 immunosorbent assay (NP-ELISA) was used to detect viral nucleoprotein (NP) in nasal swab
98 extracts from 2172 samples submitted to the AHT diagnostic laboratory as previously
99 described (Cook et al., 1988; Bryant et al., 2009b). North American samples were tested by
100 the Directigen Flu A test kit (BD) or the Binax Now Flu A test kit (Binax) as instructed by
101 the manufacturer and German samples were tested using an Optical ImmunoAssay (Viva
102 Diagnostika) also as instructed by the manufacturer (Table 1).

103

104 *2.2 Isolation of Viruses*

105 Extracts from nasopharyngeal swabs from Europe and North America that tested
106 positive by the diagnostic tests described above were cultivated in embryonated hens' eggs as
107 previously described (Bryant et al., 2009b). After the initial passage in eggs, allantoic fluid
108 was tested for virus using the haemagglutination assay (HA). If the allantoic fluid was
109 positive, the virus was used for further characterisation. If it was negative, the samples were
110 passaged through eggs again, up to 5 times in total before being declared negative (Table 1).

111

112 *2.3 Haemagglutination inhibition assay*

113 Serological analysis of paired equine sera was conducted using haemagglutination
114 inhibition assays (HI) as previously described, using either whole virus for virus
115 differentiation or ether/Tween-80 treated virus for diagnostic purposes (Daly et al., 1996).
116 For antigenic characterisation of virus isolates, untreated viruses were assayed using a panel

117 of ferret sera pre-treated with heat and periodate, using 1% chicken erythrocytes as
118 previously described (Daly et al., 1996). Geometric mean titres were calculated for three HI
119 tests for each combination. Viruses were tested against antisera specific for the strains
120 A/eq/Newmarket/1/93, A/eq/Newmarket/2/93, A/eq/Kentucky/97, A/eq/Kentucky/98,
121 A/eq/Lincolnshire/1/02, A/eq/Benelux/03, A/eq/Newmarket/5/03, A/eq/South Africa/4/03,
122 A/eq/Richmond/1/07 and A/eq/Lincolnshire/1/07 representative of European, American,
123 Florida clade 1 and Florida clade 2 viruses (Table 2).

124

125 *2.4 Viral RNA isolation, Quantitative RT-PCR, RT-PCR and sequencing*

126 Briefly, viral RNA was isolated from 140 μ L nasopharyngeal swab extract using the
127 QIAampViral RNA mini kit (Qiagen) according to the manufacturer's instructions and eluted
128 in 50 μ L. RNA standards generated by in vitro transcription from plasmid containing the
129 A/eq/Newmarket/5/03 nucleoprotein coding sequence were included alongside the
130 nasopharyngeal swab extracts. Quantitative RT-PCR was conducted using a Quantace 1 step
131 kit (Cat no. QT 205-02). Quantitative RT-PCR was carried out using a Techne Quantica
132 machine (Bryant et al., 2009a) using 2 μ L of each RNA sample was tested in duplicate wells.
133 Data were analysed using the Techne Quansoft program version 1.1.21 with a cut-off value
134 of 100 copies per well equivalent to 5.6 μ L of swab extract was used for determining positive
135 samples.

136 The HA1 gene was amplified by RT-PCR and sequenced as previously described
137 using RNA extracted directly from the nasopharyngeal swab where possible, or infected
138 allantoic fluid after passage in ovo (Bryant et al., 2009b).

139

140 *2.5 Phylogenetic trees, multiple sequence alignments and amino acid mapping*

141 To determine the genetic relationship between EIV isolates a phylogenetic tree was
142 constructed. MODELTEST was used to determine the best evolutionary model for the data
143 (<http://darwin.uvigo.es/software/modeltest.html>) (Posada and Crandall, 1998). Phylogenetic
144 trees were constructed using the PhyML software package version 2.4.5 (Guindon et al.,
145 2005; Guindon et al., 2009), with A/eq/Miami/63 as the root,
146 under the General Time Reversible substitution model (GTR + Γ_4). One hundred bootstrap
147 replicates were conducted to assess the statistical support for the tree topology. Accession
148 numbers of the virus strains in the trees can be found in the electronic supplementary data. In
149 the HA1 tree (Fig. 1) A/eq/Worcestershire/1/08 was representative of A/eq/Aboyne/1/08,
150 A/eq/Aboyne/2/08, A/eq/Hawick/1/08, A/eq/Lanarkshire/1/08, A/eq/Lanarkshire/2/08,
151 A/eq/Lanarkshire/3/08, A/eq/Lanarkshire/5/08, A/eq/Lanarkshire/6/08 and
152 A/eq/Northumberland/1/08. A/eq/Cheshire/1/09 was representative of A/eq/Cheshire/2/09.
153 A/eq/Perthshire/1/09 was representative of A/eq/Perthshire/2/09 and A/eq/Perthshire/3/09,
154 and A/eq/Dorset/1/09 was representative of A/eq/Lanarkshire/1/09 and
155 A/eq/Nottinghamshire/1/09.

156 Nucleotide substitution rates per site, per year, were estimated using the BEAST
157 package version 1.5.2 (http://beast.bio.ed.ac.uk/Main_Page) (Drummond and Rambaut,
158 2007) and the log files were analysed using Tracer version 1.4.1
159 (<http://tree.bio.ed.ac.uk/software/tracer/>). Eighty-seven HA1 sequences (1009bp) including
160 those used for the phylogenetic tree (supplementary data), isolated between 1963 and 2009
161 were used for the analysis. Estimates used the uncorrelated exponential relaxed clock model

162 (Drummond et al., 2006) and the GTR + Γ_4 model of DNA substitution. Mean results are
163 shown with the 95% highest probability density values.

164 Multiple sequence alignments were constructed using ClustalW2 (Larkin et al., 2007)
165 (<http://www.ebi.ac.uk/Tools/clustalw2/>) and Bioedit version 7.0.5
166 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>).

167

168 3. Results

169 *3.1 Isolation of viruses*

170 A total of 26 viruses were isolated from 17 EIV outbreaks the UK, 5 viruses were
171 isolated in the USA and 1 virus was isolated in Germany in 2008 to 2009 (Table 1). The
172 clinical and vaccination histories of the affected animals where available are listed in the
173 supplementary electronic data. Five further outbreaks were identified on the basis of rising
174 antibody titres but no nasal swabs were submitted.

175

176 *3.2 Sequencing and Phylogenetic analysis of the HA1 gene*

177 The HA1 gene (1009bp) from the newly characterised virus isolates was sequenced
178 and the accession numbers are listed in Table 1. In this study there were no sequence
179 differences observed within HA1 between samples extracted directly from swab material or
180 allantoic fluid from infected eggs. Phylogenetic analysis using these sequences and reference
181 strains from GenBank grouped the viruses into 5 well-supported clusters consisting of the
182 Predivergent, Eurasian and American lineages with the Florida sublineage clades 1 and 2

183 (Fig. 1). All viruses isolated and characterised from 2008 to 2009 were members of the
184 Florida sublineage clades 1 and 2. Of the outbreaks in Europe, 7 were caused by Florida
185 sublineage clade 1 viruses and 10 outbreaks were caused by Florida clade 2 viruses with
186 more clade 1 viruses being isolated in 2009 (Fig.1, Table 1) All isolates from the USA
187 grouped with the Florida clade 1 viruses. Sequence data, together with that from GenBank,
188 demonstrated an accumulation of mutations within 1009bp of HA1, with a predicted mutation
189 rate of 0.00197 (95% HPD 0.00149 - 0.00251) nucleotide substitutions per site per year from
190 1963 to 2009. This value was lower than previously estimated substitution rates of 0.00392
191 nucleotides/site/year for avian influenza virus HA (Chen and Holmes, 2006) and 0.0057
192 nucleotides/site/year for human H3 HA1 (Fitch et al., 1997) but similar to the 3.1 nucleotide
193 mutations per year for 1701 nucleotides of equine H3 based on analysis of viruses isolated
194 from 1963 to 1987 (Bean et al., 1992) with the caveat that these values were calculated using
195 different methodologies.

196 The amino acid alignment (Fig. 2) shows the substitutions between recent strains and
197 early representatives of the Florida sublineage clades 1 (A/eq/South Africa/4/03) and 2
198 (A/eq/Newmarket/5/03), the reference American lineage strain (A/eq/Newmarket/1/1993), a
199 south American strain (A/eq/Lonquen/06) (Muller et al., 2009) and sequences from the
200 viruses responsible for the Indian EIV outbreak of 2008/2009 (A/eq/Mysore/6/08,
201 A/eq/Ahmedabad/09) (Virmani et al., 2010). The characteristic amino acid substitutions of
202 A78V and S159N between the Florida clade 1 and clade 2 viruses respectively were
203 conserved in the new isolates (Fig 2., Fig.3). There were 7 further amino acid substitutions
204 within the recently isolated viruses (Fig.2). The substitution G7N found in the recent Florida
205 clade 2 viruses described here eg. A/eq/Perthshire/1/09 and A/eq/Yorkshire/3/09, was also
206 seen in Chinese and Mongolian isolates from 2007-2008 deposited in GenBank. Florida clade

207 1 viruses isolated in Europe and the USA had a G7D mutation at the same position with the
208 exception of A/eq/Virginia/1/08 and A/eq/Oklahoma/1/08. Most also had a further mutation
209 of V223I with the exception of A/eq/Oklahoma/1/08. The amino acid substitution of S47T
210 appeared in the Chinese and Mongolian Florida clade 2 isolates but was not present in the
211 European clade 2 viruses isolated to date or the Indian isolates. The R62K, D104N and
212 A138S substitutions first seen in the Florida clade 1 viruses from 2007 were present in the
213 recent isolates from Europe and the USA (Fig.2). The amino acid substitution of R135I
214 occurred in some Mongolian and Chinese isolates and was also present in
215 A/eq/Leicestershire/1/08, however it was not seen in A/eq/Perthshire/1/09 or
216 A/eq/Yorkshire/3/09 isolated later. These amino acid changes were mapped on the HA
217 structure of an avian H3N8 virus A/duck/Ukraine/63 (Ha et al., 2003) (Supplementary data
218 on line Fig. 1). The isolate A/eq/Liaoning/9/08 from China also had an amino acid
219 substitution of N165D that removes a potential *N*-glycosylation site near the head region of
220 the HA molecule. This mutation was not seen in any European or North American isolates.
221 The alignment also includes the HA1 sequence of A/eq/Lonquen/06, a virus isolated in Chile
222 in 2006 (Muller et al., 2009). It was more similar to A/eq/Newmarket/1/1993 rather than the
223 Florida sublineage strains circulating in North America and Europe. The amino acid
224 substitutions found in A/eq/Mysore/08 and A/eq/Ahmedabad/09 of V278A and Q211K were
225 not found in any Florida sublineage clade 2 viruses isolated in Europe from 2008 to 2009.

226

227 3.3 Antigenic analysis using ferret antisera

228 Virus isolates were antigenically characterised in the HI assay using ferret antisera
229 raised against 10 specific EIV strains encompassing the American and Eurasian lineages and

230 the Florida sublineage clades 1 and 2. All strains tested raised low titres against the Eurasian
231 virus antisera. The majority of the Florida clade 1 viruses, including those from the USA and
232 Europe, reacted with the clade 1 reference antisera at a titre within 2-fold of the reference
233 homologous viruses A/eq/South Africa/4/03 and A/eq/Lincolnshire/1/07 with the exception
234 of A/eq/Ohio/1/08, A/eq/Oklahoma/1/08 and A/eq/Dorset/1/09 that reacted to titres of
235 between 4 and 8-fold. These antisera reacted with the majority of the Florida sublineage
236 clade 2 viruses at a titre within 4- to 8-fold of the reference homologous viruses.
237 A/eq/Perthshire/2/09 raised a 16 to 32-fold lower titre when compared to controls, however
238 this virus raised lower titres across the panel of antisera and may be a low avidity strain.
239 Antisera raised against the Florida sublineage clade 2 viruses A/eq/Newmarket/5/03 and
240 A/eq/Richmond/1/07 reacted against the majority of recent clade 2 isolates at a titre within 2-
241 fold of the reference homologous viruses, with the exception of A/eq/Hawick/1/08 and
242 A/eq/Perthshire/1/09 that showed a 4-fold difference. The titres raised against the Florida
243 clade 1 viruses ranged from equal to and 16-fold lower when compared to the homologous
244 clade 2 reference viruses. The A/eq/Richmond/1/07 antisera raised a lower mean titre than
245 that seen with the A/eq/Newmarket/5/03 antisera when tested against the recent Florida clade
246 1 viruses. Interestingly, the antisera against the American lineage viruses
247 A/eq/Newmarket/1/93 and A/eq/Kentucky/98 raised titres within 2-fold of the homologous
248 reference viruses when tested against the majority of the recent clade 2 viruses, but titres of
249 between 4 and 32-fold lower against the recent clade 1 viruses. Antiserum raised against
250 A/eq/Kentucky/97, an early member of the Florida sublineage, generally reacted against the
251 Florida clade 2 viruses to a much higher degree than to the Florida clade 1 viruses.

252

253 4. Discussion

254 This report summarises the EIV isolates characterised at the AHT from 2008 to 2009.
255 These data show that Florida sublineage viruses from both clades 1 and 2 circulated in
256 Europe and caused sporadic disease mainly in unvaccinated horse populations. There was no
257 obvious increase in virus virulence over the past 2 years in the field, with most infected
258 individuals showing mild clinical signs followed by full recovery. In 2007, the first Florida
259 sublineage clade 1 virus was isolated in the UK (A/eq/Lincolnshire/1/07). More Florida
260 sublineage clade 1 viruses were isolated in 2009 than in 2008, which suggested clade 1
261 viruses were becoming more numerous and widespread within the United Kingdom.
262 However, it remains to be seen whether these 2 sublineages will continue to co-circulate or
263 whether one clade will become predominant. The EIV outbreaks in Lanarkshire in 2008 and
264 Dorset in 2009 began at the same time as ponies imported from the Republic of Ireland were
265 introduced to the yards. This may suggest Florida sublineage viruses from clades 1 and 2
266 were also circulating in the Republic of Ireland. The viruses isolated in Leicestershire in 2008
267 and Cheshire in 2009 were imported with animals from Holland, again suggesting both
268 Florida clades may have been circulating there. Despite the differences between the HI titres
269 of the Florida sublineage clades 1 and 2, they did not increase over time when compared to
270 2006/2007 data (Bryant et al., 2009b). This was despite there being 5 and 3 fixed amino acid
271 substitutions over this time period between the Florida clades 1 and 2, respectively (Bryant et
272 al., 2009b). However, there was up to an 8-fold difference between homologous titres for the
273 currently recommended vaccine strain A/eq/South Africa/4/03 when compared to recent
274 isolates from both Florida clades 1 and 2 (Table 2) suggesting that the current recommended
275 vaccine strain may not provide optimal protection. The most accurate method of determining
276 vaccine efficacy is by using animal challenge data, however it must be remembered that the

277 aim of the vaccine strain updates is to pre-empt any future vaccine breakdown in the field.

278 Field observations showed that the majority of infections with either Florida clade
279 were in animals that were unvaccinated or had vaccination histories that were not up to date.
280 In our limited investigation this difference was apparent on yards with mixed vaccination
281 histories such as that in Gloucestershire in 2009 where fully vaccinated competition horses
282 showed no clinical signs of infection, while unvaccinated horses on the same yard showed
283 clinical signs and seroconverted to EIV. One caveat to this was the small sample size,
284 meaning it was not possible to accurately determine the amount of vaccine breakdown
285 occurring in the field. However, RESPE (Réseau d'Epidémio-Surveillance en Pathologie
286 Equine (<http://www.respe.net/>)) and the Office International des Epizooties (OIE) World
287 Animal Health Information Database (<http://www.oie.int/>) described an ongoing large
288 outbreak in France starting in February 2009 and affecting many premises that was traced to
289 a training yard in Val de Marne, northern France. Numerous cases of vaccine breakdown
290 were reported although clinical signs were mild. The virus responsible was characterised as a
291 Florida sublineage clade 1 with similarity to A/eq/Ohio/03, similar to isolates from the UK
292 for the same period. In the USA, all the 5 characterised isolates belonged to the Florida
293 sublineage clade 1, as seen in 2006 to 2007, and were antigenically similar to the previous
294 isolates A/eq/Lincolnshire/1/07 and A/eq/California/1/07 (Bryant et al., 2009b). The authors
295 note a recent report identifying an American lineage virus in Chile with greatest similarity to
296 Argentinean isolates from the late 1990s (Muller et al., 2009) (Fig. 1, Fig. 2). There was no
297 evidence of these A/eq/Lonquen/06-like viruses circulating in North America or Europe.
298 Genetically there were 16 amino acid substitutions when compared to A/eq/Newmarket/5/03
299 (Fig. 2) so there may be some antigenically relevant changes when compared to circulating
300 strains from Europe and North America (Muller et al., 2009).

301 In mid August 2007 an outbreak of EIV was detected in a partially vaccinated horse
302 population in Japan (Ito et al., 2008; Yamanaka et al., 2008). Later that month EIV was
303 detected in a naïve horse population in Australia. EIV infection spread rapidly in both
304 countries, eventually infecting over 75,000 animals in Australia. Characterisation of the
305 representative virus A/eq/Sydney/2888-8/07 classified it as a member of the Florida
306 sublineage clade 1 viruses, typical of viruses recently isolated in North America (Callinan,
307 2008; Bryant et al., 2009b). Egypt also reported an EIV outbreak occurring from July to
308 September 2008 (<http://www.oie.int>). Sequence data from a virus isolated during the
309 outbreak suggested it was caused by a Florida clade 1 virus very similar to those circulating
310 in North America and more recently Europe, but subtly different from A/eq/Ibaraki/1/07, a
311 virus isolated in Japan during 2007 (Fig. 1, Fig 2). These three outbreaks have all since
312 resolved. It is tempting to speculate that the Florida sublineage clade 1 viruses may spread
313 east towards these regions in the future, similarly to the spread of these viruses across
314 Europe.

315 In contrast the phylogenetic tree and amino acid alignments also contain sequences
316 from China and Mongolia where thousands of equines have been infected with EIV over the
317 past 2 years (<http://www.oie.int/>) (GenBank accession numbers in supplementary materials).
318 These virus strains have been classified as Florida sublineage clade 2 viruses and are very
319 similar to those circulating in Europe (Fig.1, Fig. 2). A recent report outlining the EIV
320 outbreak in India during 2008-2009 identified the virus responsible as a Florida clade 2,
321 similar to those isolated in neighbouring China and Mongolia, suggesting that clade 2 viruses
322 are dominant in the region (Virmani et al., 2010). There are 7 amino acid substitutions
323 between the Florida sublineage clade 1 and clade 2 viruses first identified in 2006 to 2007
324 that appear to be fixed in currently circulating strains. Data in this report outlining the

325 antigenic differences between recently isolated strains has contributed to the expert
326 surveillance panel's decision to update the vaccine recommendations to include virus
327 representatives from both Florida sublineage clades for 2010. Protection may also be
328 achieved by retaining an American lineage virus (A/eq/Newmarket/1/93-like) and adding a
329 Florida clade 1 virus (A/eq/South Africa/4/03-like).

330

331 5. Conclusions

332 Florida sublineage viruses clade 1 and 2 continue to circulate in Europe while clade 1
333 viruses continue to circulate in North America. Based on antigenic analysis with ferret
334 antisera, these viruses can be readily distinguished from each other. Viruses from both clades
335 have caused extensive outbreaks around the world from 2007 to 2009 that affected thousands
336 of horses. These data contributed to the updated OIE vaccine recommendations for 2010 of
337 including both Florida clades 1 and 2 in the vaccine.

338

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347

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Table 1. EIV positives from Europe and North America 2008 to 2009

Date	Location	Lineage	Detection	Virus name	HA1 Acc.
Europe					
2/08	Wildeshausen, D	FC2	Immunoassay	A/eq/Wildeshausen/1/08	GU045273
4/08	Leicestershire, UK	FC2	ELISA	A/eq/Leicestershire/1/08	GU045280
5/08	Aboyne, UK x3	FC2	ELISA/PCR	A/eq/Aboyne/1/08	GU045271
		FC2	ELISA/PCR	A/eq/Aboyne/2/08	GU045272
5/08	Lanarkshire, UK x7	ND	RT-PCR	-	-
		FC2	ELISA/PCR/HI	A/eq/Lanarkshire/1/08	GU045275
		FC2	ELISA/PCR	A/eq/Lanarkshire/2/08	GU045276
		FC2	ELISA/PCR	A/eq/Lanarkshire/3/08	GU045277
		ND	ELISA	-	-
		FC2	ELISA	A/eq/Lanarkshire/5/08	GU045278
		FC2	ELISA/PCR	A/eq/Lanarkshire/6/08	GU045279
6/08	Hawick, UK x2	ND	ELISA	-	-
		FC2	ELISA/PCR	A/eq/Hawick/1/08	GU045274
6/08	Northumberland, UK	FC2	ELISA/PCR	A/eq/Northumberland/1/08	GU045282
6/08	Worcestershire, UK	FC2	ELISA	A/eq/Worcestershire/1/08	GU045270
6/08	Lothian, UK	FC2	ELISA/PCR	A/eq/Lothian/1/08	GU045281
3Q	Humberside, UK	ND	HI	-	-
3Q	Glamorgan, UK	ND	HI	-	-
11/08	North Wales, UK	ND	HI	-	-
2/09	Cheshire, UK	FC1	ELISA	A/eq/Cheshire/1/09	GU045285
		FC1	ELISA	A/eq/Cheshire/2/09	GU045286
4/09	Northumberland, UK	ND	ELISA	-	-
5/09	Perthshire, UK x3	FC2	ELISA/PCR	A/eq/Perthshire/1/09	GU045287
		FC2	ELISA/PCR	A/eq/Perthshire/2/09	GU045288
		FC2	ELISA/PCR	A/eq/Perthshire/3/09	-
8/09	Gloucestershire, UK	ND	HI	-	-
8/09	Kent, UK	ND	qPCR	-	-
8/09	Herefordshire, UK	FC1	ELISA/PCR	A/eq/Herefordshire/1/09	GU045269
10/09	Monmouthshire, UK	FC1	ELISA/PCR	A/eq/Monmouthshire/1/09	CY054284
10/09	Lanarkshire, UK	FC1	ELISA/PCR	A/Lanarkshire/1/09	CY054285
11/09	Nottinghamshire, UK	FC1	ELISA/PCR	A/eq/Nottinghamshire/1/09	CY054286
11/09	Dorset, UK	FC1	ELISA/PCR	A/eq/Dorset/1/09	CY054287
12/09	Perthshire, UK	ND	ELISA/PCR	A/eq/Perthshire/4/09	-
12/09	Yorkshire, UK	FC2	ELISA/PCR	A/eq/Yorkshire/1/09	-
		FC2	ELISA/PCR	A/eq/Yorkshire/2/09	-
		FC2	ELISA/PCR	A/eq/Yorkshire/3/09	CY054288
12/09	North Wales, UK	ND	HI	-	-
12/09	Bridgend, UK	FC1	ELISA/PCR	A/eq/Bridgend/1/09	CY054289
		ND	ELISA	A/eq/Bridgend/2/09	-
North America					
9/08	Ohio, USA	FC1	Directigen	A/eq/Ohio/1/08	GU045283
5/08	Oklahoma, USA	FC1	PCR	A/eq/Oklahoma/1/08	GU045284
9/08	Virginia, USA	FC1	Directigen	A/eq/Virginia/1/09	CY054291
5/09	Ohio, USA	FC1	Binax-Now	A/eq/Ohio/1/09	CY054290
2/09	Kentucky, USA	FC1	Directigen	A/eq/Kentucky/1/09	CY054292

FC1- Florida sublineage Clade 1 (A/eq/South Africa/4/03-like), FC2- Florida sublineage Clade 2 (A/eq/Newmarket/5/03-like), ELISA – Enzyme linked Immunosorbent Assay, qPCR – quantitative polymerase chain reaction, Directigen – BD diagnostics, Immunoassay – Optical ImmunoAssay Kit, Viva Diagnostika, HA1. Binax-Now – Binax, Acc. – Haemagglutinin 1 accession numbers.

Table 2. Characterisation of EIV isolates by HI assay using ferret antisera.

	Reference ferret antisera									
	N/2/93 (Eu)	Lin/1/02 (Eu)	Ben/03 (Eu)	N/1/93 (Am)	Ken/98 (Am)	Ken/97 (F)	SA/4/03 (FC1)	Lin/1/07 (FC1)	N/5/03 (FC2)	Ric/1/07 (FC2)
Reference strains										
A/eq/Newmarket/2/93	<u>128</u>	16	64	32	16	64	<8	<8	16	64
A/eq/Lincolnshire/1/02	32	<u>64</u>	256	<8	<8	32	8	16	16	16
A/eq/Benelux/03	64	<u>64</u>	<u>256</u>	<8	<8	32	8	32	16	32
A/eq/Newmarket/1/93	8	16	16	<u>128</u>	<u>128</u>	256	32	32	64	128
A/eq/Kentucky/98	16	32	32	256	<u>256</u>	512	128	128	128	512
A/eq/Kentucky/97	8	<8	<8	32	32	<u>128</u>	64	64	128	128
A/eq/South Africa/4/03	16	8	8	16	16	128	<u>1024</u>	1024	128	64
A/eq/Lincolnshire/1/07	16	8	16	16	32	64	1024	<u>512</u>	128	128
A/eq/Newmarket/5/03	32	16	16	128	128	256	128	128	<u>256</u>	256
A/eq/Richmond/1/07	32	8	8	128	128	512	128	128	512	<u>512</u>
A/eq/Pennsylvania/1/07	13	23	54	<8	11	32	32	64	23	64
Florida clade 1										
A/eq/Ohio/1/08	<8	<8	<8	<8	32	32	256	256	64	91
A/eq/Oklahoma/1/08	<8	<8	<8	<8	32	32	256	256	64	32
A/eq/Virginia/1/08	16	16	16	16	32	128	1024	512	128	128
A/eq/Cheshire/2/09	<8	8	<8	<8	8	32	256	256	64	32
A/eq/Ohio/1/09	8	8	8	8	16	64	1024	512	128	64
A/eq/Kentucky/1/09	32	32	32	32	128	256	1024	1024	256	256
A/eq/Herefordshire/1/09	16	8	8	8	16	32	512	512	128	64
A/eq/Monmouthshire/1/09	16	16	16	16	32	91	512	512	128	128
A/eq/Lanarkshire/1/09	32	32	16	45	64	256	724	1024	256	256
A/eq/Nottinghamshire/1/09	16	16	16	8	32	64	181	256	64	64
A/eq/Dorset/1/09	11	11	16	<8	64	128	128	128	16	64
Florida clade 2										
A/eq/Aboyne/1/08	8	<8	16	256	256	512	128	128	512	512
A/eq/Aboyne/2/08	<8	<8	16	128	256	512	128	256	256	512
A/eq/Hawick/1/08	8	16	16	256	256	1024	256	128	1024	1024
A/eq/Lanarkshire/1/08	<8	<8	16	256	256	512	128	128	256	512
A/eq/Lanarkshire/2/08	<8	<8	16	256	256	512	128	128	512	512
A/eq/Lanarkshire/3/08	<8	<8	16	256	128	512	128	128	256	512
A/eq/Lanarkshire/5/08	<8	8	16	256	256	512	128	128	512	512
A/eq/Lanarkshire/6/08	<8	<8	16	256	128	512	128	128	256	512
A/eq/Lothian/1/08	<8	<8	16	128	256	512	128	256	512	512
A/eq/Northumberland/1/08	8	8	16	256	128	512	256	N/A	512	512
A/eq/Worcestershire/1/08	<8	16	16	256	256	1024	128	256	512	1024
A/eq/Leicestershire/1/08	<8	8	16	256	128	768	128	64	256	256
A/eq/Wildeshausen/1/08	<8	8	16	256	128	512	128	64	256	512
A/eq/Perthshire/2/09	8	<8	<8	32	32	128	32	32	128	128
A/eq/Yorkshire/1/09	32	16	16	256	128	512	256	256	256	512
A/eq/Yorkshire/2/09	32	16	32	128	128	256	256	128	256	512

The lineage of new isolates is indicated on the left and ordered by isolation date. Homologous titres are shown in bold and underlined. N/1/93 – A/eq/Newmarket/1/93, N/2/93 – A/eq/Newmarket/2/93, Ken/97 – A/eq/Kentucky/97, Ken/98 – A/eq/Kentucky/98, Lin/1/02 – A/eq/Lincolnshire/1/02, Ben/03 – A/eq/Benelux/03, N/5/03 – A/eq/Newmarket/5/03, SA/4/03 – A/eq/South Africa/4/03, Ric/1/07 – A/eq/Richmond/1/07, Lin/1/07 – A/eq/Lincolnshire/1/07, Am – American lineage, Eu – Eurasian lineage, F – Florida sublineage, FC1 – Florida sublineage clade 1, FC2- Florida sublineage Clade 2. N/A - not available

HA1 Phylogenetic tree

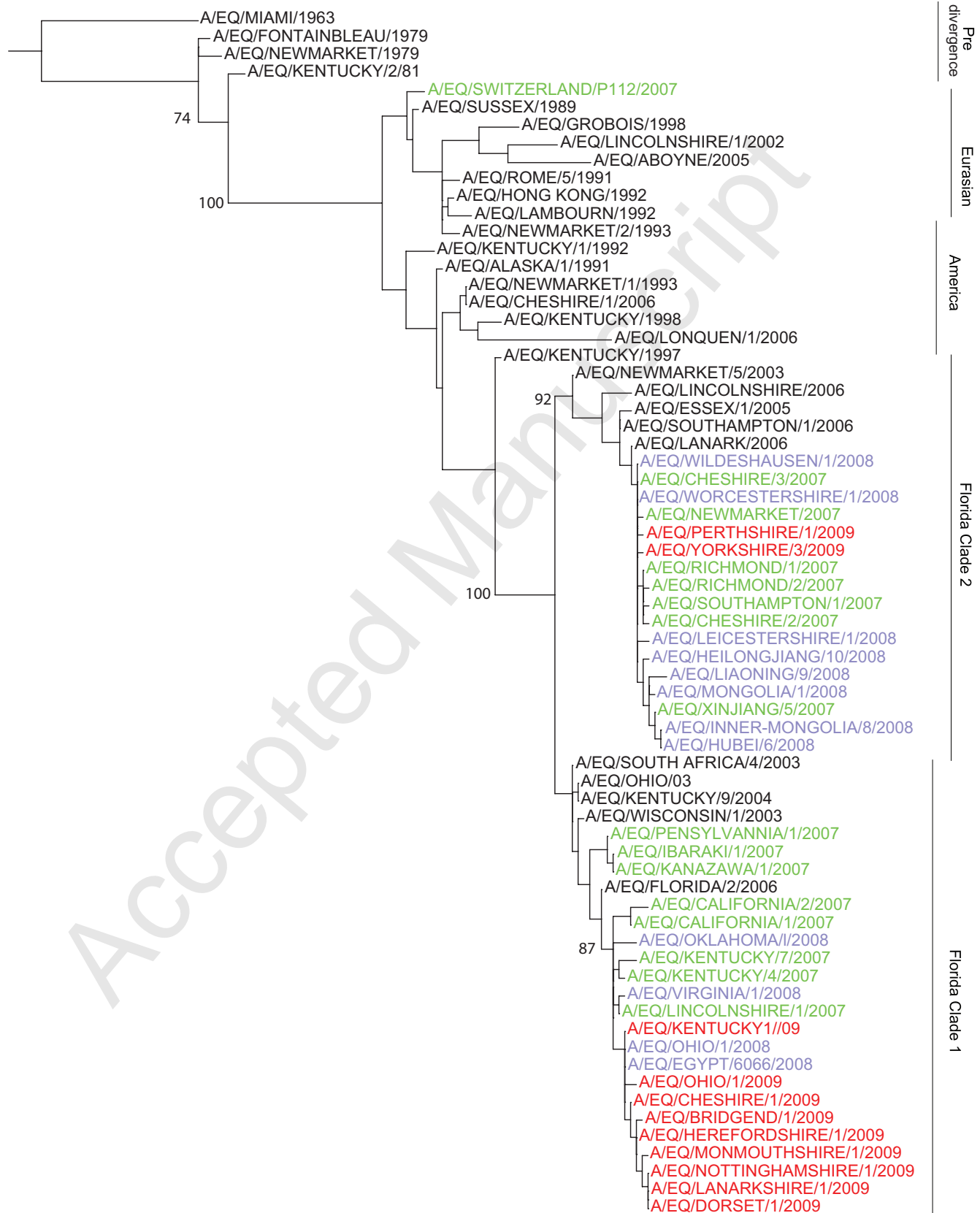


Figure 1. Phylogenetic analysis of the HA1 nucleotide sequences encoded by EIV, subtype H3N8. A maximum likelihood tree was created using PhyML version 3. Bootstrap values obtained after 100 replicates are shown at major nodes. Phylogenetic groups are shown by continuous bars on the right and are labelled as appropriate. Accession numbers for the genes reported in this manuscript and those used for reference are listed in Table 1 of 22 Sequences are coloured by date of isolation for the years 2009 (red), 2008 (blue) and 2007 (green) with the older isolates in black.

HA1 amino acid sequence alignment

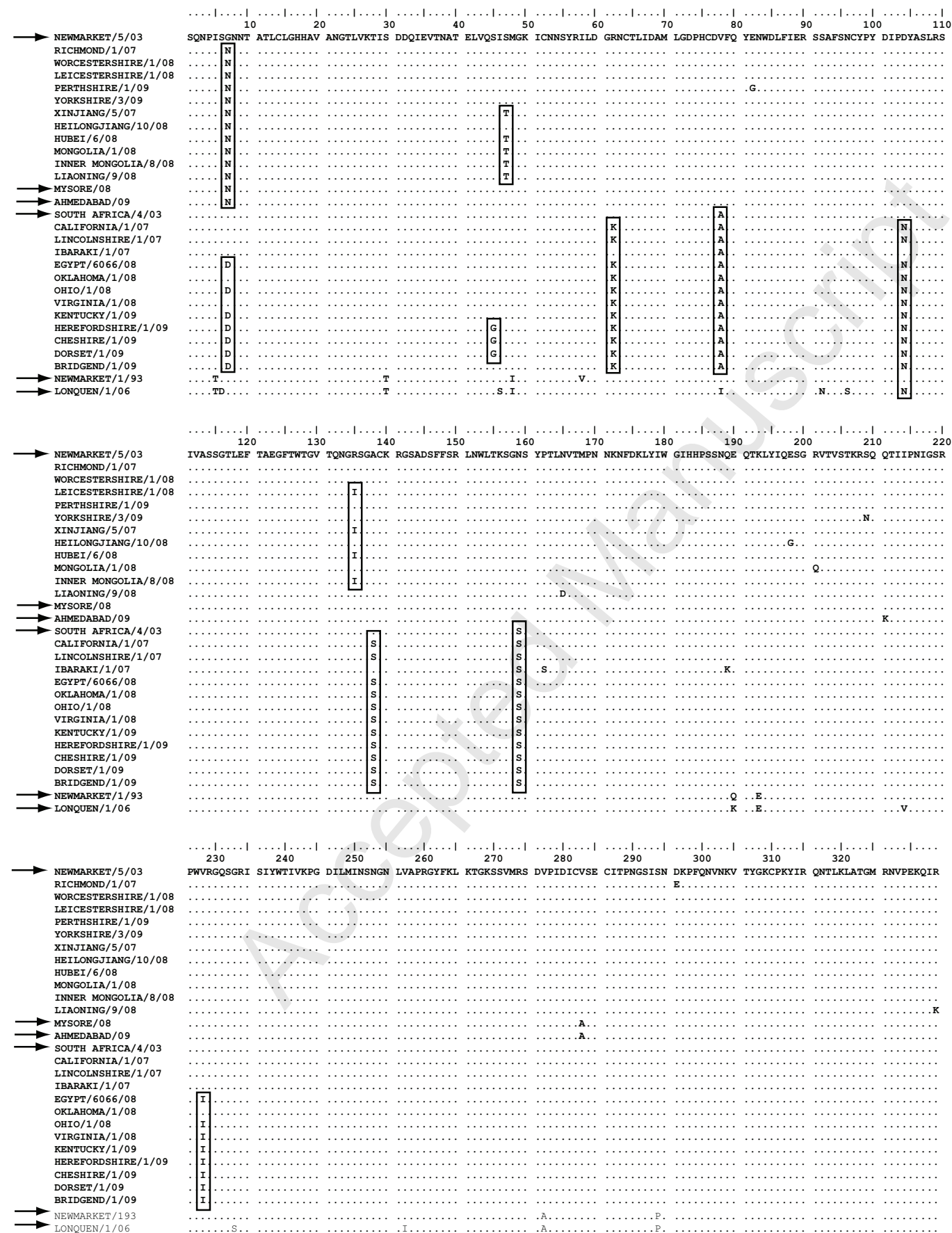


Figure 2. Predicted amino acid differences in HA1 observed between the representative strains as labelled on the left compared to A/eq/Newmarket/5/03 (top). Residues are numbered from 1 to 329 starting with the serine residue downstream of the predicted signal peptide. Amino acid identity to A/eq/Newmarket/5/03 is shown with a dot. Sequences are representative of others as described in materials and methods. Arrows highlight vaccine strains and specific examples referred to in the results.