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1 **Antigenic and genetic variations in European and North American**
2 **equine influenza virus strains isolated from 2006 to 2007**

3

4 Neil A. Bryant, Adam S. Rash, Colin A. Russell¹, Julie Ross*, Annie Cooke,
5 Samantha Bowman, Shona MacRae, Nicola Lewis¹, Romain Paillot, Reto Zanoni²,
6 Hanspeter Meier³, Lowri A. Griffiths**, Janet M. Daly***, Ashish Tiwari⁴, Thomas
7 M. Chambers⁴, J. Richard Newton, Debra M. Elton

8

9 Animal Health Trust, Centre for Preventive Medicine, Lanwades Park, Kentford, Newmarket,
10 CB8 7UU, UK,

11

12 ¹*Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ,*
13 *UK*

14 ²*Institute for Veterinary Virology, University of Bern, Laenggass-Strasse 122, CH-3012 Bern,*
15 *Switzerland*

16 ³*Equine Clinic, Department of Clinical Veterinary Medicine, Vetsuisse Faculty, University of*
17 *Bern, Laenggass-Strasse 124, CH-3012 Bern, Switzerland*

18 ⁴*Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky,*
19 *Lexington, KY 40546, USA*

20 *Present address: Department for environment, food and rural affairs, Nobel House, 17 Smith Square,
21 London, SW1P 3JR

22 **Present address: Molecular Medicine Centre, University of Edinburgh, Western General Hospital,
23 Crewe Rd, Edinburgh, EH4 2XU

24 ***Present address: Fishawack Communications Ltd, 100-102 King St, Knutsford, WA16 6HQ, UK
25

26 **Corresponding Author: Dr. Neil Bryant** Email: neil.bryant@aht.org.uk

27 Tel: +44 1638 750659 ext 1237

28 Fax: +44 1638 502461

29 **Abstract**

30 Equine influenza virus (EIV) surveillance is important in the management of equine
31 influenza. It provides data on circulating and newly emerging strains for vaccine
32 strain selection. To this end, antigenic characterisation by haemagglutination
33 inhibition assay (HI) and phylogenetic analysis was carried out on 28 equine influenza
34 virus strains isolated in North America and Europe during 2006 and 2007. In the UK,
35 20 viruses were isolated from 27 nasopharyngeal swabs that tested positive by
36 enzyme-linked immunosorbent assay. All except two of the UK viruses were
37 characterised as members of the Florida sublineage with similarity to
38 A/eq/Newmarket/5/03 (clade 2). One isolate, A/eq/Cheshire/1/06, was characterised
39 as an American lineage similar to viruses isolated up to 10 years earlier. A second
40 isolate, A/eq/Lincolnshire/1/07 was characterised as a member of the Florida lineage
41 (clade 1) with similarity to A/eq/Wisconsin/03. Furthermore, A/eq/Lincolnshire/1/06
42 was a member of the Florida sublineage (clade 2) by haemagglutinin gene (HA)
43 sequence, but appeared to be a member of the Eurasian lineage by the non-structural
44 gene (NS) sequence suggesting that reassortment had occurred.
45 A/eq/Switzerland/P112/07 was characterised as a member of the Eurasian lineage, the
46 first time since 2005 that isolation of a virus from this lineage has been reported.
47 Seven viruses from North America were classified as members of the Florida
48 sublineage (clade 1), similar to A/eq/Wisconsin/03. In conclusion, a variety of
49 antigenically distinct equine influenza viruses continue to circulate worldwide.
50 Florida sublineage clade 1 viruses appear to predominate in North America, clade 2
51 viruses in Europe.

52

53 **Keywords:** Equine Influenza virus, H3N8, surveillance, vaccine strain selection

54

55 1. Introduction

56

57 Equine influenza virus (EIV) is an *Orthomyxovirus* containing eight segments
58 of single stranded negative sense RNA encoding 10 genes. Characteristically EIV can
59 spread very rapidly in a susceptible population and continues to be an economically
60 important pathogen of horses worldwide. Typical clinical signs of infection in fully
61 susceptible animals include pyrexia, coughing, nasal discharge, dyspnoea, anorexia
62 and very rarely ataxia (Daly et al., 2006). The H7N7 subtype of EIV was first isolated
63 in 1956 in Eastern Europe (Sovinova *et al.*, 1958). However, no H7N7 subtype virus
64 has been isolated from horses since 1977 (Webster, 1993). In 1963 the first EIV from
65 the H3N8 subtype was the cause of a major epidemic in the USA that was thought to
66 have entered the country with imported horses from Argentina (Waddell et al., 1963).
67 The virus, A/eq/Miami/63, was designated as the prototype virus and viruses of this
68 subtype have circulated in horse populations ever since.

69 An early study suggested that H3N8 subtype viruses evolve as a single lineage
70 (Kawaoka et al., 1989). Later work has shown that two lineages of H3N8 subtype
71 virus emerged in the late 1980s and have continued to circulate since (Daly et al.,
72 1996). The two lineages, American and Eurasian, were initially named based on their
73 geographical location. However, it was apparent that American lineage viruses were
74 also circulating in Europe. Work by Lai and colleagues (2001) studying North
75 American EIV isolates identified a divergence of these viruses into three sublineages,
76 namely a South American lineage, a Kentucky lineage, and a Florida lineage. Further
77 evolution of the Florida sublineage has resulted in the emergence of two groups of
78 viruses with divergent HA sequences which are provisionally referred to as Florida
79 sublineage clade 1 and clade 2 viruses. Clade 1 includes the A/eq/Wisconsin/03-like

80 viruses while clade 2 is represented by the A/eq/Newmarket/5/03-like viruses (OIE,
81 2008). The Kentucky lineage has since been referred to as the classical American
82 lineage.

83 In addition to the linear evolution of HA, the segmented nature of the
84 influenza virus genome allows reassortment to take place resulting in rapid virus
85 evolution (Webster et al., 1992). Reassortment between similar viruses may occur all
86 the time but is only noticeable and significant if it occurs between distinct co-
87 circulating viral strains. RNA hybridisation experiments and nucleotide analysis of
88 H3N8 viruses have shown reassortment of RNA segments encoding NP (Bean, 1984,
89 Gorman *et al.*, 1990a), PB2 (Gorman et al., 1990b) and PA (Okazaki et al., 1989)
90 between the equine H7N7 and H3N8 subtypes.

91 The non-structural protein (NS1) has been shown to be a virulence factor in
92 influenza A virus infection *in vivo* (Hale et al., 2008) so is of particular interest when
93 studying the relative pathogenicity of isolated viruses. It functions as an antagonist of
94 the innate immune response by blocking the activation of interferon (Garcia-Sastre et
95 al., 1998) via regulatory factor-3 (Talon et al., 2000) and inhibiting the post-
96 transcriptional processing of cellular mRNAs (Fortes et al., 1994). NS1 proteins from
97 some influenza viruses are also able to inhibit cellular gene expression, inhibiting the
98 action of IFN (Hayman et al., 2006). NS1 sequences have been grouped into two
99 different alleles (A or B) (Ludwig et al., 1991). Allele A genes are found in human,
100 swine, many avian and equine viruses, with the exception of a strain from an outbreak
101 in China in 1989 (A/eq/Jilin/89), which is thought to be a relatively recent crossover
102 from an avian source and contains an allele B gene. Other allele B genes are found in
103 some American and Eurasian avian influenza viruses and a single equine
104 isolate, A/eq/Prague/56 (Suarez & Perdue, 1998).

105 New virus variants can give rise to serious EIV epidemics, such as those in
106 1979 in Europe and North America (van Oirschot et al., 1981). As a result of this
107 outbreak, mandatory vaccinations were introduced for race and competition horses in
108 the UK. However, in 1989 an outbreak of EIV occurred in the UK and continental
109 Europe caused by a virus, typified by the isolate A/eq/Suffolk/89, which infected both
110 vaccinated and non-vaccinated horses. Antigenic and sequence analysis showed this
111 virus was significantly different from the vaccine strains in use at the time (Livesay et
112 al., 1993, Binns et al., 1993). Subsequent work using a Welsh Mountain pony
113 challenge model showed that protection from virus challenge correlated with the
114 antigenic relatedness of the vaccine to the challenge virus strain (Mumford, 1998,
115 Yates & Mumford, 2000, Daly et al., 2003). OIE recommendations from 1993 state
116 that a modern virus variant should be incorporated into vaccines and that surveillance
117 efforts should be intensified to keep track of all future developments (Mumford &
118 Wood, 1993).

119 The last major outbreak of EIV in the UK was caused by a Florida sublineage
120 clade 2 virus, designated A/eq/Newmarket/5/03, that caused disease in vaccinated and
121 non-vaccinated horses in the field (Lai et al., 2001, Newton et al., 2006). Sporadic
122 outbreaks of EIV have continued in the UK, Ireland and continental Europe and much
123 larger outbreaks have occurred in Sweden (International Thoroughbred Breeders
124 International Collating Centre (ICC) administered by AHT,
125 <http://www.aht.org.uk/icc/linksicc.html>), Japan, Northern China, Mongolia and
126 Australia (Office International des Epizooties (OIE), World Animal Health
127 Information Database, <http://www.oie.int/>). The EIV outbreaks in Japan and Australia
128 in 2007 were attributable to closely related Florida sublineage clade 1 viruses, with

129 similarity to A/eq/Wisconsin/03 and were probably of North American origin
130 originally (Yamanaka et al., 2008, Callinan, 2008).

131 In 2006 the OIE officially recommended that vaccines should contain a
132 representative of the Florida lineage (clade 1) (A/eq/SouthAfrica/4/03 or
133 A/eq/Ohio/03) and a representative of the Eurasian lineage (A/eq/Newmarket/2/93-
134 like) (OIE, 2006). Recommendations are reviewed on an annual basis and take into
135 account up to date surveillance data from a number of countries. The previous
136 recommendation was confirmed by the OIE in 2008 following its annual review of
137 events in 2007 (OIE, 2008). This report is a summary of the EIV surveillance data
138 collected at the Animal Health Trust in 2006 and 2007, including isolates submitted
139 from the UK, Switzerland and the USA consisting of information on sequence
140 analysis, antigenic and phylogenetic relationships.

141

142 **2. Materials and methods**

143

144 *2.1 NP-ELISA and Directigen™Flu A*

145

146 A nucleoprotein enzyme-linked immunosorbent assay (NP-ELISA) was used
147 to detect viral nucleoprotein (NP) in the nasal swab extract (Cook et al., 1988).
148 Briefly, 100 µl Tween-20 treated nasal swab extract (2µl 10% Tween-20 in 200 µl
149 nasal wash for 5 min) was added to a 96-well plate coated with anti-A/eq/Sussex/89
150 rabbit polyclonal antibody and allowed to bind for 90 min. After washing 3 times in
151 phosphate buffered saline-0.2%-Tween-20 (PBS-T), bound virus was incubated with
152 the mouse monoclonal antibody 3G9, specific for NP, in a 60 min incubation step,
153 then washed three times in PBS-T. Samples were then incubated with anti-mouse

154 peroxidase conjugated IgG antibody (Dako) for 30 min incubation step at 37 °C
155 followed by three washes, before being developed with the chromogen (3'3'5'5'-
156 Tetramethylbenzidine) TMB (Sigma) for up to 5 minutes at room temperature. The
157 ELISA was stopped using 2M sulphuric acid and plates were analysed
158 spectrophotometrically at 450 nm.

159 North American samples were tested by the Directigen™ Flu A test kit (BD,
160 NJ, USA) as instructed by the manufacturer.

161

162 *2.2 Viruses*

163

164 *2.2.1 Isolation of viruses from the UK*

165

166 Nasopharyngeal swabs (made in house) were taken from horses showing signs
167 of acute respiratory disease. Swabs were placed in sterile tubes containing virus
168 transport medium (VTM) consisting of PBS, 200 U/ml streptomycin, 150 U/ml
169 penicillin, 5µg/ml fungizone (Gibco) and 600 µg/ml tryptone phosphate broth and
170 chilled on ice immediately after collection. VTM samples that tested positive in the
171 NP-ELISA were inoculated into the allantoic cavities of 10 day old embryonated
172 hens' eggs. Eggs were incubated at 34°C and harvested 3 days post infection. Virus
173 was detected by HA assay using 1% chicken erythrocytes in PBS. Viruses were
174 serially passaged until stable titres were obtained, up to a maximum of five passages
175 (Table 1).

176

177 *2.2.2 Viruses from North American and Switzerland*

178

179 Viruses were isolated as described in eggs. Infected allantoic fluid was
180 received frozen on dry ice. In order to obtain enough material for haemagglutination
181 inhibition assays and sequencing, viruses were further amplified in eggs.

182

183 *2.3 Haemagglutination inhibition assay*

184

185 Serological analysis was conducted using haemagglutination inhibition assays
186 (HI) as previously described using either native virus for virus differentiation or
187 ether/Tween-80 treated virus for diagnostic purposes (Daly et al., 1996). For virus
188 differentiation the untreated viruses were assayed using ferret sera pre-treated with
189 heat and periodate, using 1% chicken erythrocytes as previously described. Geometric
190 mean titres were calculated for three HI tests for each combination. Viruses were
191 tested against antisera specific for the strains A/eq/Newmarket/1/93,
192 A/eq/Newmarket/2/93, A/eq/Kentucky/97, A/eq/Kentucky/98,
193 A/eq/Lincolnshire/1/02, A/eq/Benelux/03, A/eq/Newmarket/5/03 and A/eq/South
194 Africa/4/03.

195

196 *2.4 Viral RNA isolation, RT-PCR and sequencing*

197

198 Viral RNA was isolated from nasopharyngeal swabs or allantoic fluid using
199 the QIAamp Viral RNA mini kit (Qiagen) according to the manufacturer's
200 instructions. Genes of interest were amplified using the RobusT I RT-PCR kit
201 (Finnzymes) with 2µl of template RNA and 0.1 U/ml RNAsin (Promega) using the
202 gene specific primers H3HA1/1 (5'-AGCAAAAGCAGGGGATATTC) and
203 H3HA1/2 (5'-GCTATTGCTCCAAAGATTC) for HA1 and primers NS1/EcoI (5'-

204 CGGAATTCAGCAAAAAGCAGGGTGACAAA) and NS1/Xba2 (5'-
205 GCTCTAGAAGTAGAAACAAGGGTGTTCATC) for NS1 at a final
206 concentration of 0.2 μ M. Samples were incubated 30 min at 48°C and 2 min at 94°C
207 for the RT reaction, followed by 5 min at 94°C, 1 min at 55°C and 4 min at 72°C for
208 30 cycles. PCR products were analysed on a 1% agarose gel stained with ethidium
209 bromide and purified using the QIAquick PCR Purification Kit (Qiagen) according to
210 the manufacturer's instructions. PCR products were sequenced using ABI BigDye®
211 Terminator v3.1 (Applied Biosystems) according to manufacturer's instructions on an
212 ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). Nucleotide sequences
213 were visualized and edited using Seqman II version 5.03 (DNASTAR Inc).

214

215 *2.5 Phylogenetic trees, multiple sequence alignments and amino acid mapping*

216

217 To determine the relationship between the EIV isolates a phylogenetic tree
218 was constructed. MODELTEST was used to determine the best evolutionary model
219 for the data (<http://hcv.lanl.gov/content/sequence/findmodel/findmodel.html>) (Posada
220 & Crandall, 1998). Phylogenetic trees were constructed using PAUP version 4.0
221 (Swofford, 1999) under the General Time Reversible substitution model, as
222 determined by MODELTEST, with branch swapping by tree-bisection-reconnection.
223 One hundred bootstrap replicates were conducted to assess the statistical support for
224 the tree topology. Accession numbers of the virus strains in the trees are as follows
225 (HA1, NS1): A/eq/Miami/63 (M29257, CY028840), A/eq/Fontainebleau/79
226 (CY032405, CY032409), A/eq/Newmarket/79 (D30677, AF001662),
227 A/eq/Kentucky/2/81 (CY028820, CY028824), A/eq/Sussex/89 (X85090, FJ375212),
228 A/eq/Rome/5/91 (D30684, AF001669), A/eq/Hong-Kong/92 (L27597, AF001670),

229 A/eq/Lambourn/92 (X85087, AF001672), A/eq/Newmarket/2/93 (X85089,
230 FJ375211), A/eq/Grobois/98 (AY328471, FJ195452), A/eq/Lincolnshire/02
231 (FJ195450, FJ195447), A/eq/Aboyne/05 (EF541442, FJ195444),
232 A/eq/Kentucky/1/92 (CY030149, CY030153), A/eq/Alaska/1/91 (CY030157,
233 AF001667), A/eq/Kentucky/98 (AF197241, FJ195446), A/eq/Newmarket/1/93
234 (X85088, FJ375210), A/eq/Kentucky/97 (AF197249, FJ195445), A/eq/South
235 Africa/4/03 (Prof. Alan Guthrie, Personal Communication), A/eq/Kentucky/9/04
236 (FJ195451, FJ195448), A/eq/Ohio/03 (DQ124192, DQ124186), A/eq/Wisconsin/1/03
237 (DQ222913, DQ222917), A/eq/Newmarket/5/03 (FJ375213, FJ375209),
238 A/eq/Essex/1/05 (EF541439, FJ195449). In the HA1 tree (Fig. 1a)
239 A/eq/Southampton/1/07 was representative of A/eq/Southampton/2/07 and
240 A/eq/Solihull/2/07, A/eq/Cheshire/3/07 was representative of A/eq/Horsham/07,
241 A/eq/Berkshire/07 and A/eq/Maidstone/2/07, A/eq/Richmond/1/07 was representative
242 of A/eq/Maidstone/1/07, A/eq/Solihull/1/07 and A/eq/Strathaven/07. In the NS1 tree
243 (Fig. 1b) A/eq/Richmond/1/07 was representative of A/eq/Strathaven/07,
244 A/eq/Maidstone/1/07, A/eq/Richmond/2/07, A/eq/Solihull/1/07 and
245 A/eq/Solihull/2/07, A/eq/Southampton/1/07 was representative of
246 A/eq/Southampton/2/07.

247 Multiple amino acid sequence alignments were obtained using Bioedit version
248 7.0.5. (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Protein structures were
249 visualised and individual amino acids were identified using FirstGlance in Jmol
250 (<http://molvis.sdsc.edu/fgij/index.htm>).

251

252 3. Results

253

254 *3.1 Isolation of EIV from the UK*

255

256 A total of 27 NP-ELISA positive nasopharyngeal swabs were received in 2006
257 and 2007 from the UK, and from 20 of these, virus was recovered following growth in
258 eggs (Table 1). The following outlines the clinical and vaccination histories of the
259 affected animals.

260 In 2006, A/eq/Cheshire/1/06 was isolated from a large mixed yard of horses of
261 different ages. At least 10 animals were infected and showed typical equine influenza
262 (EI) clinical signs, two of which were recently vaccinated. A/eq/Southampton/1/06
263 was isolated from one of four infected horses that had uncertain vaccination histories.
264 The NP-ELISA positive horse from Stowmarket in the UK was unvaccinated but had
265 travelled from Ireland 2 days prior to showing typical clinical signs of EI.
266 A/eq/Lanark/1/06 was isolated from a horse with an uncertain vaccination history that
267 was imported from Poland three days before showing clinical signs of infection.
268 A/eq/Lincolnshire/1/06 was isolated from an unvaccinated British spotted pony that
269 had been in contact with a group of recently imported Friesian horses. Clinical signs
270 were typical and included cough, nasal discharge and reduced appetite.

271 In 2007, A/eq/Horsham/1/07 was isolated from an infected horse on a large
272 dressage yard. The horse had an uncertain vaccination history but had recently been
273 imported from Holland. Clinically it had pyrexia of 41.4°C, nasal discharge and
274 dyspnoea. The positive HI test from Stoke-on-Trent was from a non-vaccinated 6
275 year old gelding which presented with a marked pyrexia of 40.6°C, anorexia, profuse
276 serous nasal discharge, frequent harsh dry cough, lymphadenopathy and mild ataxia.
277 The onset of clinical signs was reported 4-5 days earlier. Four foci of EIV infection
278 in the Midlands, Kent and Hampshire were identified from the end of May to the end

279 of June 2007 (Solihull, Maidstone, Southampton). Nasopharyngeal swabs were taken
280 from horses typically showing clinical signs of pyrexia with rectal temperatures up to
281 41°C/, profuse and frequently mucopurulent nasal discharge and frequent, harsh, dry
282 cough with substantially increased respiratory rates. In all four outbreaks the index
283 cases were animals that were recently imported into the country, having been bought
284 at a horse sale in County Kilkenny in the Republic of Ireland on the 26th May. All
285 these horses were reported to either be non-vaccinated or of unknown vaccination
286 history. There was one case of vaccine breakdown where a 6 year old Irish sports
287 horse resident at the Solihull yard showed clinical signs having previously been
288 vaccinated with Equilis Prequenza TE™ (Intervet) on 13th April 2007 and 8th May
289 2007. A/eq/Strathaven/1/07 was isolated from a 13 year-old, non-vaccinated Shetland
290 pony with typical EI clinical signs, inappetance and inco-ordination. Nine further
291 ponies were reported to be affected. This pony was on premises where horses had
292 returned back sick from the Royal Highland Show near Edinburgh. There were no
293 mandatory vaccination requirements for horses attending the show and many of them
294 were consequently not vaccinated. The horse from Hawick which tested positive by
295 NP-ELISA was shipped over from Irish horse sales around 14th August 2007. It was
296 vaccinated with Equip FT on 10th April 2006 and 8th May 2006 and then with Prevac
297 Pro™ (Intervet) on 11th November 2006. Clinical signs of inappetance, pyrexia,
298 cough, mucopurulent nasal discharge, swollen lymph nodes and lethargy were
299 observed. The positive NP-ELISA from Stowmarket was an 8 year-old pony mare,
300 last vaccinated with Prevac T Pro™ (Intervet) 12th July 2005 showed clinical signs
301 consisted of pyrexia of 39°C with unilateral nasal discharge and lethargy.
302 A/eq/Lincolnshire/107 was isolated from a horse with clinical signs imported into the
303 UK from Spain. The lorry had been elsewhere in Europe prior to this and there were

304 no reported case of influenza in horses on the yard in Spain from where it came. Two
305 in contact animals had clinical signs of influenza subsequently and one was identified
306 as NP ELISA positive. Three separate viruses were isolated from Cheshire. The first,
307 a 2 ½ year old gelding presented with a very mild pyrexia of 38.4°C with slight
308 mucopurulent nasal discharge. Several in contact horses were coughing and had
309 mucopurulent nasal discharges with mild pyrexia. At the second site, 9 horses were
310 affected and there were 47 in contact horses. Vaccinated animals appeared healthy
311 throughout. At the third site a disease outbreak occurred on a large livery yard that
312 was restricted to unvaccinated young horses. A/eq/Berkshire/1/07 was recovered from
313 a vaccinated horse newly imported from Holland although the exact vaccine was
314 unknown. The two positive NP-ELISAs from Lambourn were from vaccinated
315 national hunt horses. The first horse received ProteqFlu™ (Merial) for the last 3
316 vaccinations and was last boosted August 1st 2007 (prior to this was 6th January 2007).
317 The second horse received Equilis Prequenza TE™ (Intervet) as the last vaccination
318 on 28th June 2007, ProteqFlu™ (Merial) before that on January 6th 2007 and Duvaxyn
319 IE-plus™ (Fort Dodge) before that on 13th June 2006. A/eq/Newmarket/1/07 was
320 isolated from an infected horse from one of three affected training yards. The animal
321 had shown only mild signs and had been most recently vaccinated with ProteqFlu™
322 (Merial).

323

324 3.2. Genetic characterisation

325

326 3.2.1. HAI

327

328 The HA1 (1009 bp) from each virus isolate was sequenced and accession
329 numbers are listed in table 1. EIV isolates were grouped into five well supported
330 clades consisting of the Pre-divergent, Eurasian and American lineages with the
331 Florida sublineage clades 1 and 2 (Fig. 1a). Phylogenetic analysis grouped 18 of the
332 20 isolates from the UK within the Florida clade 2 sublineage. The isolate designated
333 A/eq/Lincolnshire/1/07 was unique among the UK isolates as it clustered with the
334 Florida clade 1 sublineage viruses. The isolate designated A/eq/Cheshire/1/06
335 grouped with the American (Kentucky) lineage viruses. The isolate from Switzerland
336 was different from those isolated in the UK over the same time period as it belonged
337 to the Eurasian lineage and clustered alongside the older strain A/eq/Sussex/89 (Fig
338 1a). The HA1 of the North American virus isolates characterised here all clustered in
339 the Florida clade 1 sublineage, typical of the viruses isolated in or originating from
340 North America (Lai et al., 2001) (Fig. 1a).

341

342 3.2.2 NS1

343

344 The NS1 (692 bp) coding sequence of each virus isolate was determined and
345 accession numbers are listed in Table 1. The topology of the maximum-likelihood tree
346 showed four clades corresponding to the Pre-divergent, American, Eurasian and
347 Florida lineage viruses similar to those seen with the HA1 coding sequences. There
348 was evidence of further differentiation into two different clades in the Florida
349 sublineage cluster, as seen with HA1, the bootstrap value for this division in NS1 was
350 81% compared to 100% for HA1 (Fig.1b). However, A/eq/Lincolnshire/1/06, which
351 was within the Florida sublineage when analysing the HA1 coding sequence, grouped
352 with the Eurasian lineage when analysing the NS1 coding sequence, and

353 A/eq/Kentucky/98, which was American lineage by HA1 phylogeny was more similar
354 to the Florida clade 1 viruses by NS1 phylogeny (Fig. 1b). The two isolates that were
355 unusual according to their HA1 sequences, A/eq/Cheshire/1/06 and
356 A/eq/Switzerland/P112/07, grouped as their HA1 sequences did, with the American
357 lineage and Eurasian lineage NS1 sequences respectively. Both phylogenetic trees in
358 Figure 1 contain only unique sequences and any isolates with identical sequences
359 were omitted as described in the materials and methods.

360

361 *3.3 Antigenic analysis of EIV isolates*

362

363 The antigenic properties of the EIV isolates were evaluated using ferret
364 antisera raised against a panel of 8 AHT reference strains (Table 2). The isolate
365 A/eq/Berkshire/1/07 was not characterised by HI assay as it only grew to a low titre in
366 eggs after repeated passage. The American lineage and Florida sublineage clade 1 and
367 2 viruses isolated in the UK and North America exhibited low reactivity to the
368 antisera raised against Eurasian lineage isolates with the exception of
369 A/eq/Pennsylvania/1/07, for which the highest titre obtained was against the
370 A/eq/Benelux/03 antisera, although titres were generally low across the entire
371 reference panel. The 17 Florida sublineage clade 2 viruses isolated from the UK and
372 tested by HI all showed similar patterns of reactivity with the antisera, with 16 of the
373 17 strains showing an increase of between 2 and four fold in reactivity against
374 A/eq/Newmarket/5/03 when compared to A/eq/South-Africa/4/03, and one strain
375 showing a greater than 4 fold increase. Conversely 5 of the 7 Florida clade 1 viruses,
376 one of which was isolated in the UK (A/eq/Lincolnshire/1/07) and the rest from North
377 America, reacted 4-fold or more higher against A/eq/South-Africa/4/03 when

378 compared to A/eq/Newmarket/5/03. The 2 remaining viruses, namely
379 A/eq/California/2/07 and A/eq/Pennsylvania/1/07 both reacted higher to the
380 A/eq/South-Africa/4/03 but only to a level of 2-fold or less respectively. Another
381 trend between the clade 1 and clade 2 Florida sublineage viruses was their reactivity
382 with the antiserum raised against A/eq/Kentucky/97, an early member of the Florida
383 sublineage. The majority of Clade 1 viruses raised relatively low titres (≤ 32) against
384 A/eq/Kentucky/97, with the exception of A/eq/Kentucky/4/07 and
385 A/eq/Kentucky/7/07, whereas the Clade 2 viruses raised higher titres of between 91
386 and 512. In contrast to the Florida sublineage clade 2 viruses isolated in the UK,
387 A/eq/Cheshire/1/06, classified as an American lineage virus, had relatively low
388 reactivity with the A/eq/Kentucky/97 antiserum and is more similar to those titres
389 seen with the Florida clade 1 viruses from North America. It also reacted weakly
390 across the panel when compared to A/eq/Newmarket/1/93, even though the predicted
391 HA1 amino acid sequence is identical.

392 A/eq/Switzerland/P112/07, classified as a member of the Eurasian lineage on
393 the basis of HA1 sequence, had low reactivity across the panel. It raised a weak titre
394 of 16 against A/eq/Benelux/03 and A/eq/Kentucky/97 and was a low reactor to all
395 sera.

396

397 *3.4 Amino acid alignments*

398

399 In order to identify which amino acids may play a role in the separation into
400 different phylogenetic clades, amino acid sequence alignments of the HA1 and NS1
401 coding sequences were constructed with representative isolates aligned against
402 A/eq/Newmarket/5/03. Any HA1 changes are summarised in Table 3, NS1 changes

403 are highlighted in Table 4. The numbering of the amino acid positions of the HA1
404 sequences starts with the serine residue immediately downstream of the predicted
405 signal peptide cleavage site as 1. This was consistent with the numbering used for the
406 H3 HA structure (Ha et al., 2003). Negative numbers represent the predicted signal
407 sequence. All the recent isolates had the substitutions at amino acids 190 and 193
408 when compared to A/eq/Newmarket/1/93, predicted to be within antigenic site B and
409 first observed in A/eq/Newmarket/5/03 with the exception of A/eq/Cheshire/1/06 that
410 was more similar to A/eq/Newmarket/1/93. The viruses belonging to the Florida
411 sublineage clade 1 (North American isolates A/eq/Florida/2/06, A/eq/California/2/07,
412 A/eq/Kentucky/4/07, A/eq/Kentucky/7/07, A/eq/Pennsylvania/1/07 and the UK
413 isolate A/eq/Lincolnshire/1/07) all had very similar HA1 sequences in which the
414 majority differ from American and Eurasian lineage viruses at residues -16, 62, 78,
415 104, 138 and 159. Between A/eq/South-Africa/4/03 and the latest Florida sublineage
416 clade 1 isolates there were 10 different amino acid substitutions, one of which was in
417 the signal sequence. The isolates A/eq/California/2/07 and A/eq/Kentucky/7/07 both
418 contained 6 of these amino acid substitutions. A/eq/Pennsylvania/1/07 and
419 A/eq/Ibaraki/07 shared the substitutions at residues V78A and N159S but not the
420 changes at residues R62K, D104N or A138S with the other clade 1 isolates. They also
421 had the additional unique changes at residues P162S and Q189K and when compared
422 to A/eq/Newmarket/5/03. The Florida sublineage clade 2 viruses isolated in the UK
423 including, A/eq/Lincolnshire/1/06, A/eq/Richmond/1/07 and A/eq/Newmarket/1/07,
424 had a unique two amino acid insertion in the predicted signal peptide at residues -11
425 and -10, including one aromatic residue, combined with a leucine to phenylalanine
426 substitution at -9, to generate a 'FIF' or 'FNF' motif. The predicted HA1 sequences
427 of A/eq/Newmarket/1/93 and A/eq/Cheshire/06 were identical, with substitutions at

428 residues -2, 5, 30, 48, 58, 190, 193, 272 and 289 when compared to
429 A/eq/Newmarket/5/03. The Eurasian lineage viruses A/eq/Sussex/89 and
430 A/eq/Switzerland/P112/07 shared many of the amino acid changes listed above for the
431 American lineage viruses, but also have some specific changes at T163I, K261R and
432 I276T.

433 NS1 changes are summarised in Table 4. The previously observed truncation
434 with a stop codon at position 220 was found in all 2006 to 2007 isolates apart from
435 A/eq/Cheshire/1/06 and A/eq/Switzerland/P112/07. There were at least 8 amino acid
436 substitutions seen between the NS1 from American (A/eq/Newmarket/1/93 and
437 A/eq/Cheshire/1/06) and Eurasian lineage viruses (A/eq/Sussex/89 and
438 A/eq/Switzerland/P112/07). There was also no stop codon at position 220 and the
439 protein terminated at position 231. A/eq/Lincolnshire/1/06 NS1 also had these
440 substitutions, making it most similar to the Eurasian lineage viruses, but with a further
441 7 substitutions unique to this isolate. Further substitutions between Florida sublineage
442 clade 2, American and Eurasian lineage viruses occur consistently at I48S and I84V,
443 and more recently H206Y. The differences between the Florida clade 2 sublineage
444 viruses and the other lineages highlight A/eq/Lincolnshire/1/07 as a Florida clade 1
445 virus.

446

447 **4. Discussion**

448

449 EIV surveillance was conducted in order to identify and characterise currently
450 circulating and new emerging virus strains to provide data for vaccine strain selection.
451 The surveillance data for the UK and Europe was consistent with the previous
452 observations that there was a predominance of Florida sublineage clade 2 viruses in

453 circulation (Newton et al., 2006, Damiani et al. 2007). The index cases of the
454 outbreaks that occurred in June 2007 in the UK were animals that had been recently
455 imported into England, having been bought at a horse sale in County Kilkenny in the
456 Republic of Ireland. It was therefore likely to be at least partially representative of the
457 viruses circulating in the Republic of Ireland at that time (Newton et al., 2007).
458 Florida sublineage clade 1 viruses were isolated in North America. Interestingly
459 outbreaks of disease often follow horse movement and introduction into new
460 environment. This may be due to stress associated with travelling making the animal
461 more susceptible to infection.

462 The topology of the maximum-likelihood tree of the HA1 data was similar to
463 that previously described (Fig. 1a) (Daly et al., 1996, Lai et al., 2001) and showed that
464 viruses from the two Florida sublineage clades 1 and 2 continued to co-circulate in
465 2006 and 2007. Amino acid alignment of 2003 Florida clade 1 and clade 2 virus
466 isolates suggested that the changes V78A and N159S were consistent between the
467 clades (Table 3.). They also identified an insertion within the signal peptide that
468 created a 'FIF' motif at position -11 to -9 in the Florida sublineage clade 2 viruses
469 isolated in the UK. This insertion was first seen in isolates from 2004 and has been
470 seen in all UK isolates belonging to clade 2 since then (eg. A/eq/Wales/1/05,
471 accession number EF541438). The authors note that the HA sequence of a virus
472 isolated during the Mongolian outbreak in 2008 has been released to GenBank that
473 indicates that this belongs to the Florida clade 2 sublineage (Accession number
474 AB436910). The amino acid alignment in table 3 shows an isoleucine to an
475 asparagine residue substitution at position -10 in A/eq/Newmarket/1/07, the last 2007
476 isolate described in this paper that changes the 'FIF' sequence to 'FNF'. This change

477 was not seen in the Mongolian isolate suggesting this virus had a greater similarity to
478 the older UK clade 2 isolates (data not shown).

479 A/eq/Cheshire/1/06 was characterised as a member of the American lineage,
480 viruses of which have not been detected in the UK since isolation of
481 A/eq/Moulton/98, A/eq/Snailwell/98 and A/eq/Edinburgh/98 (Newton et al., 1999).
482 A/eq/Switzerland/P112/07 was the first virus to be characterised as a Eurasian lineage
483 virus, since the isolate from Scotland in 2005 designated A/eq/Aboyne/05 (Accession
484 number: EF541442). The lack of isolates belonging to these other lineages may be
485 due to the relatively small numbers of samples received or the Eurasian lineage may
486 be dying out. It also raises the possibility that circulating Florida sublineage virus
487 strains may be able to acquire gene segments from a simultaneously circulating
488 Eurasian strain or *vice versa*, potentially resulting in changes in pathogenicity or
489 antigenicity. Reassortment such as this may be responsible for the Eurasian lineage-
490 like NS1 found in A/eq/Lincolnshire/1/06, classified as a Florida sublineage clade 2
491 virus based on HA1 sequence. A/eq/Cheshire/1/06 and A/eq/Switzerland/P112/07
492 appeared to be more closely related to viruses isolated between 1989 and 1993 rather
493 than the recent Florida sublineage. There are reports of viruses being isolated that
494 appear to date from earlier in time, and it has been suggested it is due to a
495 phenomenon called frozen evolution (Endo et al., 1992, Borchers et al. 2005). This
496 may result from a reduced amount of antigenic drift compared to the majority of
497 circulating EIVs. However, these viruses are also very similar to virus reference
498 strains A/eq/Newmarket/1/93 and A/eq/Sussex/89, suggesting a laboratory escape or
499 vaccine origin as suggested previously (Gupta et al., 1993). The majority of vaccines
500 currently available, especially in the UK, contain these older strains so they should
501 provide good protection against infection. However, the presence of these viruses in

502 the field in addition to the Eurasian and Florida lineage viruses complicates matters
503 when trying to determine the best possible combination of EIV strains for future
504 vaccine recommendations.

505 As the UK and North America have two of the few active surveillance
506 systems, it is not known which are the most widely circulating viruses globally and
507 certain phylogenetic lineages may be over represented. Horse movement is a major
508 contributing factor in transmitting EIV around the world, despite mandatory
509 vaccination and quarantine procedures. Epidemiological and phylogenetic studies of
510 EIV outbreaks have often found direct links with international horse movement such
511 as in South Africa in 1986 (Guthrie et al., 1999), Hong Kong in 1992 (Lai et al.,
512 1994) and Newmarket in 2003 (Newton et al., 2006). EIV was responsible for several
513 large outbreaks of respiratory disease in 2007. In mid August 2007 an outbreak of
514 EIV was detected in a partially vaccinated horse population in Japan (Yamanaka *et*
515 *al.*, 2008). Table 3 includes the partial sequence of A/eq/Ibaraki/07, a virus
516 representative of the outbreak strain (Yamanaka et al., 2008). Horses were vaccinated
517 with an inactivated vaccine containing A/eq/LaPlata/93 (American lineage),
518 A/eq/Avesta/93 (Eurasian lineage) and A/eq/Newmarket/77 (H7N7). These strains,
519 although dated, are not unlike the strains used in the vaccines available in the UK (Fig
520 1). This raised the concern that currently available vaccines may also be vulnerable to
521 breakdown when used to protect against this or a related virus, as seen in 2003 with
522 the emergence of A/eq/Newmarket/5/03 which infected both vaccinated and non-
523 vaccinated equids in Europe. A/eq/Ibaraki/07 had highest sequence identity with
524 A/eq/Pennsylvania/1/07, a Florida sublineage clade 1 like virus with an HA1
525 sequence typical of those circulating in North America. However, the HI data
526 presented in table 2 shows that sera raised against currently used vaccine strains and

527 those recommended by the OIE (OIE, 2008) raise relatively low titres to
528 A/eq/Pennsylvania/1/07. It remains to be determined whether this virus represents a
529 new clade within the evolution of EIV or whether representative strains are still
530 circulating in the field. The HA1 amino acid alignments show substitutions P162S
531 and Q189K which are not found in the other clade 1 viruses described in this report.
532 A/eq/South-Africa/4/03 virus that reacts highly to antisera raised against
533 A/eq/Kentucky/98 (Table 2) and to antisera raised against itself only differs at these
534 two amino acids within HA1, suggesting these two changes may be responsible for
535 the differences in antigenicity observed. Residue 189 maps adjacent to the receptor-
536 binding pocket on the structure of H3 and was exposed on the surface (Ha et al.,
537 2003). The substitution P162S has been previously observed in some pre-divergent
538 strains such as A/eq/Tokyo/71 (Accession number: M24720) but this is on a
539 substantially different backbone sequence that only has 85.8% sequence identity
540 within HA1 when compared to A/eq/Newmarket/5/03. The isolate
541 A/eq/California/2/07 also had low reactivity to the antisera in table 2. This strain
542 contained 6 amino acid substitutions when compared to A/eq/South Africa/4/03, of
543 which R62K, T192K and K207E mapped at or near the surface of the HA1 molecule
544 when aligned with the three dimensional structure of H3 HA (Ha et al., 2003) (data
545 not shown). Later in the same month in 2007 there was an outbreak of EIV in
546 Australia, this time mainly in an unvaccinated population after the virus escaped from
547 a quarantine station into the general horse population. Subsequently the two outbreaks
548 have been linked and a representative virus from the Australian outbreak
549 (A/eq/Sydney2888-8/07) was almost identical to A/eq/Ibaraki/07 (Callinan, 2008,
550 Yamanaka et al., 2008). The introduction of horse movement restrictions and horse
551 free buffer zones in conjunction with vaccination using ProteqFlu™ (Merial) which

552 expressed both A/eq/Kentucky/94 and A/eq/Newmarket/2/93 HA, finally eradicated
553 EIV from Australia.

554 Currently there are two recommended components to the vaccine, a Eurasian
555 isolate and a Florida sublineage clade 1 isolate (OIE bulletin, 2008). Many vaccine
556 manufacturers have yet to update their vaccines as recommended by the OIE,
557 although recently 2 manufacturers have done so with the inclusion of A/eq/Ohio/03
558 (ProteqFlu™, Merial and Calvenza™, Boeringher Ingelheim). As the antigenic
559 distance increases between vaccine strains and those circulating, so does the risk of
560 EIV infection in the field associated with vaccine breakdown. If both Florida
561 sublineage virus clades persist and diverge in the field, vaccines will have to be
562 updated to allow both clades to be represented in order to confer clade specific
563 immunity.

564 Vaccine studies have shown that currently available vaccines reduce virus
565 shedding and clinical signs when compared to control ponies when challenged with
566 recent strains of EIV (Paillot et al., 2008, Bryant et al., 2008). However, in these
567 studies, ponies were challenged two weeks post vaccination under optimal
568 experimental conditions more accurately modelling vaccination in the face of
569 infection. Vaccine breakdown in the field did occur in Japan among racehorses
570 previously vaccinated 3 months before the outbreak (Yamanaka et al., 2008). Other
571 experiments in ponies have shown protection induced by non-adjuvanted vaccines
572 containing virus from one lineage against challenge infection with heterologous virus
573 is reduced when compared to challenge with the homologous virus, as in the case of
574 vaccination with A/eq/Newmarket/2/93 (Eurasian) and subsequent challenge with
575 A/Newmarket/1/93 (American). Interestingly, this lack of protection occurs in one
576 direction only in that vaccination with A/eq/Newmarket/1/93 does protect against

577 challenge with A/eq/Newmarket/2/93 (Daly et al., 2004), suggesting the American
578 lineage isolates may confer some protection against Eurasian isolates in the future.

579 Sequencing of the NS segment showed the truncation that was previously
580 observed in A/eq/Kentucky/5/02 (Accession number AY855345) (Quinlivan et al.,
581 2005) and then in A/eq/Newmarket/5/03 in the UK (Table 4). The NS1 protein has
582 been shown to play a role in virus pathogenicity. Differences in pathogenicity have
583 been observed between Eurasian and American lineage viruses in experimental
584 infection of ponies (Yates & Mumford, 2000, Paillot et al., 2006) and it is possible
585 NS1 plays a role similar to that seen for other influenza A viruses. However, the role
586 of specific amino acid substitutions between the NS1 proteins of Eurasian and
587 American lineage EIV isolates have yet to be elucidated.

588 In conclusion, 28 EIV strains from North America and Europe isolated in
589 2006–2007 were characterised in detail, in order to aid the vaccine strain selection
590 process. Viruses belonging to the American (Kentucky), Eurasian and Florida
591 sublineages have been isolated with varying degrees of antigenic drift occurring from
592 vaccine strains. The majority of isolates belonged to the Florida sublineage, sequence
593 divergence and antigenic differences support the further division of this group into 2
594 clades. To date, Florida clade 1 viruses have been isolated in North America with
595 outbreaks in Japan and Australia likely to be due to horse movements. Clade 2 viruses
596 continue to predominate in Europe. This has reinforced the importance of continued
597 surveillance in the field in order to identify any new emerging threats to the equine
598 industry.

599

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610

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748 **Figure legends**

749

750 **Figure 1:** Phylogenetic analyses of the HA1 (a) and NS1 (b) nucleotide sequences
751 encoded by EIV, subtype H3N8. Maximum likelihood trees were created
752 using PAUP version 4.0. Bootstrap values obtained after 100 replicates are
753 shown at the major nodes. Phylogenetic groups are shown by continuous bars
754 on the right and are labeled as appropriate. Accession numbers for the genes
755 reported in this manuscript are listed in table 1 and representative sequences
756 are shown on the trees by an asterisk (*) as described in materials and methods.
757 Reference strains used in the trees are listed in materials and methods.

758

Figure 1

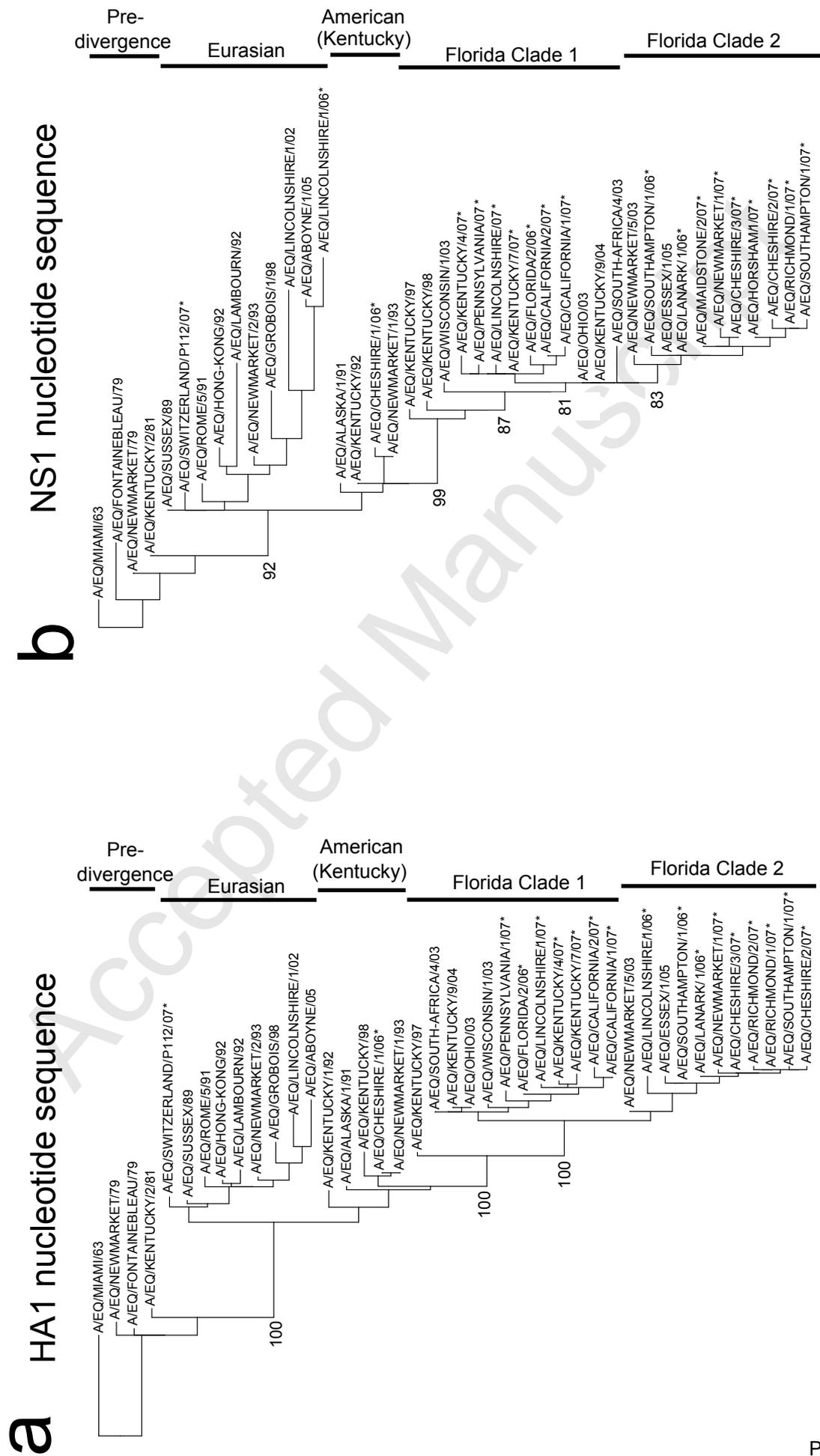


Table 1. EIV isolates from Europe and North America 2006-2007

Date	Location	Lineage	Detection	Virus name	HA1	NS1
United Kingdom virus Isolates						
03/06	Cheshire, UK	Am	ELISA/PCR	A/eq/Cheshire/1/06	FJ195401	FJ195423
04/06	Southampton, UK	F C2	ELISA/PCR	A/eq/Southampton/1/06	FJ195391	FJ195418
07/06	Stowmarket, UK	ND	ELISA	-	-	-
08/06	Lanark, UK	F C2	ELISA/PCR	A/eq/Lanark/1/06	FJ195400	FJ195419
10/06	Lincolnshire, UK	F C2	ELISA/PCR	A/eq/Lincolnshire/06	FJ195399	FJ195420
02/07	Horsham, UK	F C2	ELISA/PCR	A/eq/Horsham/07	FJ195411	FJ195421
05/07	Stoke-on-Trent, UK	F C2	HI	-	-	-
06/07	Solihull, UK	F C2	ELISA/PCR	A/eq/Solihull/1/07	FJ195414	FJ195437
06/07	Solihull, UK	F C2	ELISA/PCR	A/eq/Solihull/2/07	FJ195415	FJ195438
06/07	Solihull, UK	ND	ELISA	-	-	-
06/07	Solihull, UK	ND	ELISA	-	-	-
06/07	Maidstone, UK	F C2	ELISA/PCR	A/eq/Maidstone/1/07	FJ195413	FJ195439
06/07	Maidstone, UK	F C2	ELISA/PCR	A/eq/Maidstone/2/07	FJ195412	FJ195428
06/07	Southampton, UK	F C2	ELISA/PCR	A/eq/Southampton/1/07	FJ195394	FJ195430
06/07	Southampton, UK	F C2	ELISA/PCR	A/eq/Southampton/2/07	FJ195416	FJ195440
06/07	Strathaven, UK	F C2	ELISA/PCR	A/eq/Strathaven/1/07	FJ195417	FJ195441
08/07	Hawick, UK	ND	ELISA	-	-	-
08/07	Stowmarket, UK	ND	ELISA	-	-	-
09/07	Lincolnshire, UK	F C1	ELISA/PCR	A/eq/Lincolnshire/1/07	FJ195398	FJ195427
11/07	Richmond, UK	F C2	ELISA/PCR	A/eq/Richmond/1/07	FJ195395	FJ195429
11/07	Richmond, UK	F C2	ELISA/PCR	A/eq/Richmond/2/07	FJ195396	FJ195442
11/07	Cheshire, UK	F C2	ELISA/PCR	A/eq/Cheshire/1/07	FJ195410	FJ195443
11/07	Cheshire, UK	F C2	ELISA/PCR	A/eq/Cheshire/2/07	FJ195392	FJ195425
11/07	Cheshire, UK	F C2	ELISA/PCR	A/eq/Cheshire/3/07	FJ195393	FJ195426
11/07	Berkshire, UK	F C2	ELISA/PCR	A/eq/Berkshire/1/07	FJ195409	-
11/07	Lambourn, UK	F C2	ELISA	-	-	-
11/07	Lambourn, UK	F C2	ELISA	-	-	-
12/07	Newmarket, UK	F C2	ELISA/PCR	A/eq/Newmarket/1/07	FJ195397	FJ195424
Swiss virus isolate						
11/07	Switzerland	Eu	EGG	A/eq/Switzerland/P112/07	FJ195408	FJ195422
North American virus isolates						
5/06	Florida	F C1	Directigen	-	-	-
10/06	Florida	F C1	Directigen	A/eq/Florida/2/06	FJ195403	FJ195433
1/07	Kentucky	F C1	Directigen	A/eq/Kentucky/4/07	FJ195404	FJ195434
7/07	Kentucky	F C1	Directigen	A/eq/Kentucky/7/07	FJ195405	FJ195435
9/07	Pennsylvania	F C1	Directigen	A/eq/Pennsylvania/1/07	FJ195406	FJ195436
-/07	California	F C1	Directigen	A/eq/California/1/07	FJ195407	FJ195431
-/07	California	F C1	Directigen	A/eq/California/2/07	FJ195402	FJ195432

Eu – Eurasian lineage, Am - American lineage, F C1- Florida sublineage Clade 1 (A/eq/Wisconsin/03-like), F C2- Florida sublineage Clade 2 (A/eq/Newmarket/5/03-like), ELISA – Enzyme linked Immunosorbent Assay, PCR – Polymerase chain reaction, Directigen – BD diagnostics, MD, USA, HA1 – Haemagglutinin 1 accession numbers, NS1 – non-structural protein 1 accession numbers.

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

		Reference Ferret Antisera							
		N/1/93 (Am)	N/2/93 (Eu)	Ken/97 (F C1)	Ken/98 (Am)	Lin/02 (Eu)	Ben/03 (Eu)	N/5/03 (F C2)	SA/4/03 (FC1)
Reference viruses	A/eq/Newmarket/1/93	128	8	256	128	10	13	81	20
	A/eq/Newmarket/2/93	40	81	102	32	81	81	20	8
	A/eq/Kentucky/97	64	<8	256	64	8	8	203	51
	A/eq/Kentucky/98	256	8	406	256	20	20	128	32
	A/eq/Lincoln/1/02	<8	23	54	8	128	256	16	8
	A/eq/Benelux/03	8	64	64	16	203	256	20	8
	A/eq/Newmarket/5/03	91	8	362	91	8	11	362	91
	A/eq/South Africa/4/03	16	<8	102	256	8	8	81	406
American	A/eq/Cheshire/1/06	32	<8	32	23	<8	8	32	16
Florida Clade 1	A/eq/Lincolnshire/1/07	16	<8	64	<8	<8	8	64	256
	A/eq/Florida/2/06	8	<8	32	16	<8	8	45	256
	A/eq/California/1/07	<8	<8	32	16	<8	<8	32	128
	A/eq/California/2/07	<8	<8	32	8	<8	<8	16	32
	A/eq/Kentucky/4/07	11	<8	91	32	8	8	91	512
	A/eq/Kentucky/7/07	64	<8	128	64	12	16	128	1024
	A/eq/Pennsylvania/1/07	<8	13	32	11	23	54	23	32
Florida Clade 2	A/eq/Southampton/1/06	64	<8	128	64	8	11	256	91
	A/eq/Lincolnshire/1/06	91	<8	362	128	8	11	64	32
	A/eq/Lanark/1/06	64	8	362	102	10	13	203	81
	A/eq/Cheshire/1/07	128	<8	362	128	16	23	724	128
	A/eq/Cheshire/2/07	45	<8	181	64	<8	8	128	45
	A/eq/Cheshire/3/07	32	<8	128	32	<8	8	128	45
	A/eq/Horsham/1/07	64	<8	256	45	<8	<8	91	45
	A/eq/Maidstone/1/07	91	<8	256	128	<8	<8	256	128
	A/eq/Maidstone/2/07	256	<8	512	128	16	16	256	128
	A/eq/Strathaven/1/07	128	<8	512	256	8	16	512	128
	A/eq/Richmond/1/07	64	<8	256	128	<8	8	256	64
	A/eq/Richmond/2/07	64	<8	256	128	<8	8	256	64
	A/eq/Solihull/1/07	64	<8	256	64	<8	<8	128	64
	A/eq/Solihull/2/07	64	<8	128	64	<8	8	128	45
	A/eq/Southampton/1/07	91	<8	256	64	<8	8	128	45
	A/eq/Southampton/2/07	128	<8	256	128	8	8	181	64
A/eq/Newmarket/1/07	64	<8	181	64	<8	8	128	32	
Eurasian	A/eq/Switzerland/P112/07	<8	<8	16	<8	8	16	<8	<8

Lineage of new isolates is indicated on the left. Homologous titres are shown in bold. 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/02 – A/eq/Lincolnshire/1/02, Ben/03 – A/eq/Benelux/03, N/5/03 – Newmarket/5/03, SA/4/03 – A/eq/South Africa/4/03, Am – American lineage, Eu – Eurasian lineage, FC1 – Florida sublineage clade 1, FC2- Florida sublineage Clade 2.

Table 3. Amino acid alignment of predicted HA1 sequences compared to A/eq/Newmarket/5/03.

STRAIN	-16	-11	-10	-9	-8	-2	5	7	26	30	48	58	62	78	104	105	138	158	159	162	163	189	190	192	193	207	213	244	261	265	272	273	275	276	289	291
NEW/5/03	K	^	^	L	I	A	I	G	V	S	M	I	R	V	D	Y	A	G	N	P	T	Q	E	T	K	K	I	M	K	S	V	P	D	I	S	D
LIN/06	.	F	I	F	.	.	.	D	I	K	.	.	M	.	G
SOU/1/06	.	F	I	F	.	.	.	D	M
RIC/1/07	.	F	I	F	.	.	.	N	E
RIC/2/07	.	F	I	F	M	.	.	N	E
MAI/2/07	.	F	I	F	.	.	.	N
NEW/07	.	F	N	F	.	.	.	N
LIN/07	T	K	A	N	.	S	.	S
SA/4/03	A	.	.	S	.	S
FLO/2/06	T	A	.	.	S	.	S
CAL/2/07	T	K	A	N	.	S	.	S	K	.	E
KEN/4/07	T	K	A	N	H	S	.	S
KEN/7/07	T	K	A	N	.	S	.	S	L	.	N	.	.
PEN/07	T	A	S	S	.	.	K
IBA/07	N/A	A	S	S	.	.	K
NEW/1/93	V	T	.	.	T	I	V	Q	.	E	A	.	.	.	P	.
CHE/06	V	T	.	.	T	I	V	Q	.	E	A	.	.	.	P	.
SUS/89	V	T	.	.	T	I	V	I	K	.	.	.	E	V	.	R	.	A	L	G	T	P	.
SWI/07	V	T	.	.	T	I	V	E	.	.	I	K	.	.	.	E	V	T	R	.	A	.	.	T	P	.	

Residues are numbered from 1 to 328 starting with the serine residue downstream of the predicted signal peptide (1-17aa) cleavage site. Amino acid identity to A/eq/Newmarket/5/03 (Accession Number: FJ375213) is shown with a dot. Other reference strains are A/eq/South-Africa/4/03 (Prof. Alan Guthrie, Personal communication), A/eq/Ibaraki/07 (Accession number: AB360549), A/eq/Newmarket/1/93 (Accession Number: X85088) and A/eq/Sussex/89 (Accession Number: X85090). A/eq/Richmond/1/07 is representative of A/eq/Cheshire/1/07, A/eq/Cheshire/2/07, A/eq/Maidstone/1/07, A/eq/Solihull/1/07, A/eq/Solihull/2/07, A/eq/Strathaven/1/07, A/eq/Horsham/1/07, A/eq/Lanark/1/06, A/eq/Southampton/1/07 and A/eq/Southampton/2/07; A/eq/Lincolnshire/1/07 is representative of A/eq/California/1/07; A/eq/Newmarket/1/07 is representative of A/eq/Berkshire/1/07, and A/eq/Cheshire/3/07. N/A – not available; ^ - not present.

Table 4. Amino acid alignments of the predicted NS1 sequences compared to A/eq/Newmarket/5/03.

STRAIN	5	22	44	48	53	56	59	66	71	76	77	84	86	95	96	111	124	140	156	179	180	185	194	206	210	212	213	216	220	227	228	230	231		
NEW/5/03	T	F	R	I	D	T	H	E	K	A	L	I	T	L	D	V	I	I	V	G	V	L	I	H	G	P	S	S	*	K	P	I	*		
SOU/1/06	*
NEW/07	*
LIN/07	.	.	.	S	V	R	*	
RIC/1/07	Y	*	
SOU/1/07	F	Y	*	
CHE/1/07	P	Y	*	
SA/4/03	.	V	.	S	V	I	*	
FLO/2/06	.	.	.	S	V	.	.	.	I	*	
CAL/1/07	.	.	.	S	V	*	
KEN/4/07	.	.	.	S	.	.	K	.	.	F	V	*	
PEN/07	.	.	.	S	V	.	.	G	*	
NEW/1/93	.	.	K	S	N	.	R	.	E	.	.	V	A	F	.	.	S	.	P	R	E	.	V	.	*		
CHE/06	.	.	K	S	N	.	R	.	D	.	.	V	A	F	.	.	S	.	P	R	E	.	V	.	*		
SUS/89	.	.	K	S	.	.	R	.	E	.	.	V	A	V	.	.	.	P	R	E	.	V	.	*	
LIN/06	.	.	K	S	N	I	R	.	E	E	.	V	.	.	.	V	K	.	.	E	I	F	V	Y	.	.	T	P	R	E	.	V	.	*	
SWI/07	.	.	K	S	.	.	R	.	E	.	F	V	A	V	.	.	.	P	R	E	S	V	.	*	

Amino acid residue identity to A/eq/Newmarket/5/03 is shown with a dot. Stop codons are represented with an asterisk (*). Residues are numbered from the *N*-terminal methionine. A/eq/California/1/07 is representative of A/eq/California/2/07 and A/eq/Kentucky/7/07; A/eq/Southampton/1/07 is representative of A/eq/Southampton/2/07; A/eq/Newmarket/1/07 is representative of A/eq/Cheshire/3/07, A/eq/Maidstone/2/07, A/eq/Lanark/1/06 and A/eq/Horsham/1/07; A/eq/Richmond/1/07 is representative of A/eq/Cheshire/2/07, A/eq/Strathaven/1/07, A/eq/Richmond/2/07, A/eq/Solihull/1/07 and A/eq/Solihull/2/07.