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

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Abstract

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

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

1. Introduction

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

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

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

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

2. Materials and methods

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

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

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

2.2 Isolation of Viruses

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

2.3 Haemagglutination inhibition assay

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

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

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

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

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

2.5 Phylogenetic trees, multiple sequence alignments and amino acid mapping

To determine the genetic relationship between EIV isolates a phylogenetic tree was constructed. MODELTEST was used to determine the best evolutionary model for the data (http://darwin.uvigo.es/software/modeltest.html) (Posada and Crandall, 1998). Phylogenetic trees were constructed using the PhyML software package version 2.4.5 (Guindon et al., 2005; Guindon et al., 2009), with 59 nucleotide sequences and A/eq/Miami/63 as the root, under the General Time Reversible substitution model (GTR + Γ_4). One hundred bootstrap replicates were conducted to assess the statistical support for the tree topology. Accession numbers of the virus strains in the trees can be found in the electronic supplementary data. In the HA1 tree (Fig. 1) A/eq/Worcestershire/1/08 was representative of A/eq/Aboyne/1/08, A/eq/Aboyne/2/08, A/eq/Hawick/1/08, A/eq/Lanarkshire/1/08, A/eq/Lanarkshire/2/08, A/eq/Lanarkshire/3/08, A/eq/Lanarkshire/5/08, A/eq/Lanarkshire/6/08 and A/eq/Northumberland/1/08. A/eq/Cheshire/1/09 was representative of A/eq/Cheshire/2/09. A/eq/Perthshire/1/09 was representative of A/eq/Perthshire/2/09 and A/eq/Perthshire/3/09, and A/eq/Dorset/1/09 was representative of A/eq/Lanarkshire/1/09 and A/eq/Nottinghamshire/1/09.

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

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

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

3. Results

3.1 Isolation of viruses

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

3.2 Sequencing and Phylogenetic analysis of the HA1 gene

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

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

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

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

3.3 Antigenic analysis using ferret antisera

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

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

4. Discussion

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

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

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

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

5. Conclusions

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

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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/Wideshausen/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
		ND	RT-PCR	-	-
5/08	Lanarkshire,UK x7	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
		ND	ELISA	-	-
6/08	Hawick, UK x2	FC2	ELISA/PCR	A/eq/Hawick/1/08	GU045274
		ND	ELISA	-	-
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/Perthsire/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	**128**	16	64	32	16	64	<8	<8	16	64
A/eq/Lincolnshire/1/02	32	**64**	256	<8	<8	32	8	16	16	16
A/eq/Benelux/03	64	64	**256**	<8	<8	32	8	32	16	32
A/eq/Newmarket/1/93	8	16	16	**128**	128	256	32	32	64	128
A/eq/Kentucky/98	16	32	32	256	**256**	512	128	128	128	512
A/eq/Kentucky/97	8	<8	<8	32	32	**128**	64	64	128	128
A/eq/South Africa/4/03	16	8	8	16	16	128	**1024**	1024	128	64
A/eq/Lincolnshire/1/07	16	8	16	16	32	64	1024	**512**	128	128
A/eq/Newmarket/5/03	32	16	16	128	128	256	128	128	**256**	256
A/eq/Richmond/1/07	32	8	8	128	128	512	128	128	512	**512**
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

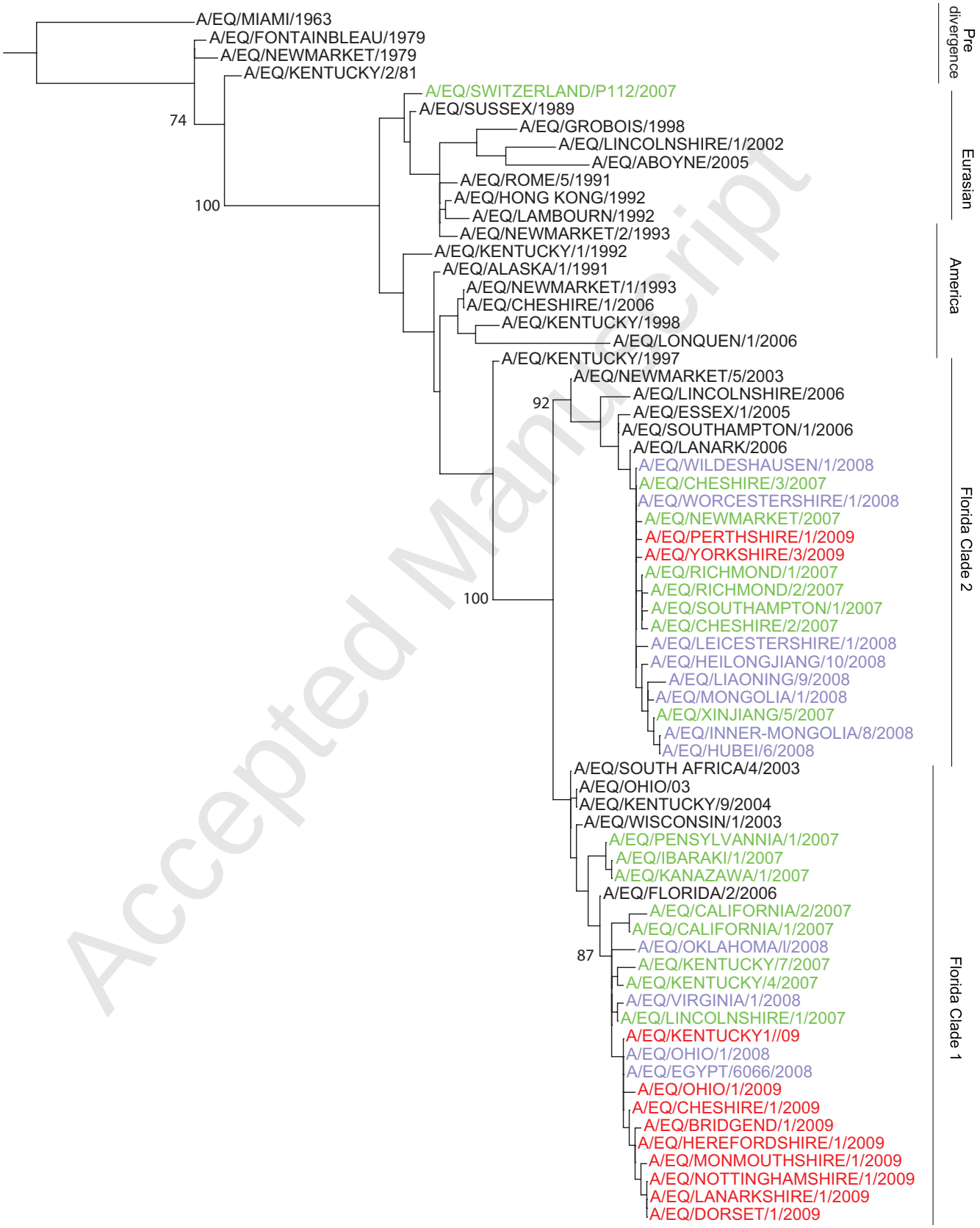

Figure 1. Phylogenetic analysis of the HA1 nucleotide sequences emcoded by EIV, subtype H3N8. A maximum likelihood tree was created usimg PhyML version 3. Bootstrap values obtained after 100 replicates are shown at major nodes. Phylogenetic groups are shown by continuous bars on the right and are labelled as appropriate. Accession numbers for the genes reported in this manuscript and those used for reference are listed in Table 1. 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

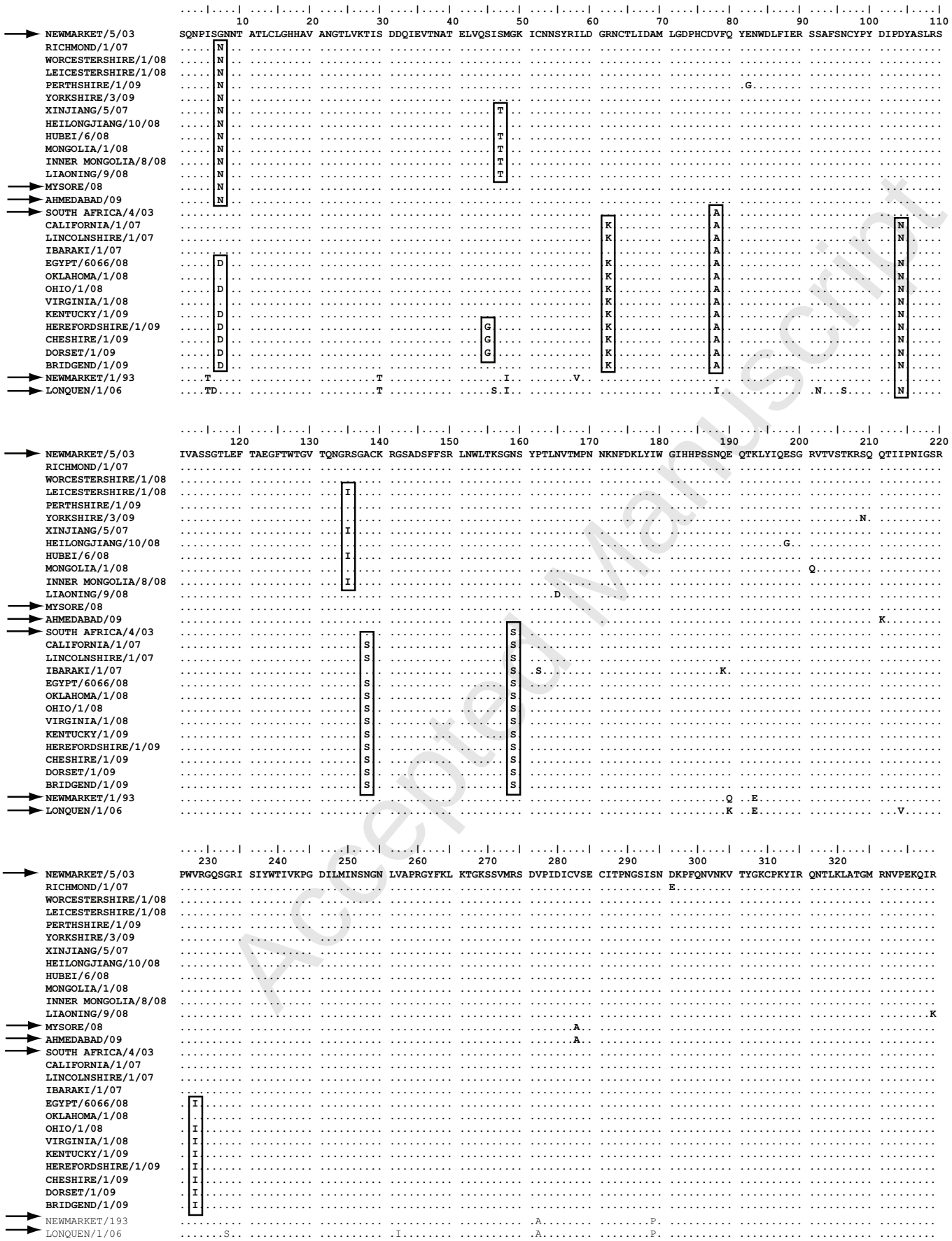

Figure 2. Predicted amino acid differences in HA1 observed between the representative strains as labelled on the left compared to A/eq/Newmarket/5/03 (top). Residues are numbered from 1 to 329 starting with the serine residue downstream of the predicted signal peptide. Amino acid identity to A/eq/Newmarket/5/03 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.