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Antigenic and genetic variations in European and North American equine influenza virus strains (H3N8) isolated from 2006 to 2007

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ABSTRACT

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

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1. Introduction

Equine influenza virus (EIV) is an *Orthomyxovirus* containing eight segments of single stranded negative sense RNA encoding 10 genes. Characteristically EIV can spread very rapidly in a susceptible population and continues to be an economically important pathogen of horses worldwide. Typical clinical signs of infection in fully susceptible animals include pyrexia, coughing, nasal

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discharge, dyspnoea, anorexia and very rarely ataxia (Daly et al., 2006). The H7N7 subtype of EIV was first isolated in 1956 in Eastern Europe (Sovinova et al., 1958). However, no H7N7 subtype virus has been isolated from horses since 1977 (Webster, 1993). In 1963 the first EIV from the H3N8 subtype was the cause of a major epidemic in the USA that was thought to have entered the country with imported horses from Argentina (Waddell et al., 1963). The virus, A/eq/Miami/63, was designated as the prototype virus and viruses of this subtype have circulated in horse populations ever since.

An early study suggested that H3N8 subtype viruses evolve as a single lineage (Kawaoka et al., 1989). Later work has shown that two lineages of H3N8 subtype virus emerged in the late 1980s and have continued to circulate since (Daly et al., 1996). The two lineages, American and Eurasian, were initially named based on their geographical location. However, it was apparent that American lineage viruses were also circulating in Europe. Work by Lai et al. (2001) studying North American EIV isolates identified a divergence of these viruses into three lineages, namely a South American lineage, a Kentucky lineage, and a Florida lineage later referred to as the Florida sublineage. Further evolution of the Florida sublineage has resulted in the emergence of two groups of viruses with divergent HA sequences which are provisionally referred to as Florida sublineage clades 1 and 2 viruses. Clade 1 includes the A/eq/Wisconsin/03-like viruses while clade 2 is represented by the A/eq/Newmarket/5/03-like viruses (OIE, 2008). The Kentucky lineage has since been referred to as the classical American lineage.

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

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

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

The last major outbreak of EIV in the UK was caused by a Florida sublineage clade 2 virus, designated A/eq/Newmarket/5/03, that caused disease in vaccinated and non-vaccinated horses in the field (Lai et al., 2001; Newton et al., 2006). Sporadic outbreaks of EIV have continued in the UK, Ireland and continental Europe and much larger outbreaks have occurred in Sweden (International Thoroughbred Breeders International Collating Centre (ICC) administered by AHT, http://www.aht.org.uk/icc/linksicc.html), Japan, Northern China, Mongolia and Australia (Office International des Epizooties (OIE), World Animal Health Information Database, http://www.oie.int/). The EIV outbreaks in Japan and Australia in 2007 were attributable to closely - related Florida sublineage clade 1 viruses, with similarity to A/eq/Wisconsin/03 and were probably of North American origin originally (Yamanaka et al., 2008; Callinan, 2008).

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

2. Materials and methods

2.1. NP-ELISA and Directigen™ Flu A

A nucleoprotein (NP) enzyme-linked immunosorbent assay (NP-ELISA) was used to detect viral nucleoprotein in the nasal swab extract (Cook et al., 1988). Briefly, 100 μl Tween-20 treated nasal swab extract (2 μl 10% Tween-20 in 200 μl nasal wash for 5 min) was added to a 96-well plate coated with anti-A/eq/Sussex/89 rabbit polyclonal antibody and allowed to bind for 90 min. After washing 3

times in phosphate buffered saline-0.2%-Tween-20 (PBS-T), bound virus was incubated with the mouse monoclonal antibody 3G9, specific for NP, in a 60 min incubation step, then washed three times in PBS-T. Samples were then incubated with anti-mouse peroxidase conjugated IgG antibody (Dako) for 30 min incubation step at 37 °C followed by three washes, before being developed with the chromogen (3′3′5′5′-Tetramethylbenzidine) TMB (Sigma) for up to 5 min at room temperature. The ELISA was stopped using 2 M sulphuric acid and plates were analysed spectrophotometrically at 450 nm.

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

2.2. Viruses

2.2.1. Isolation of viruses from the UK

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

2.2.2. Viruses from North American and Switzerland

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

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/1/06	FJ195399	FJ195420
02/07	Horsham, UK	F C2	ELISA/PCR	A/eq/Horsham/1/07	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 GROWTH	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.

2.3. Haemagglutination inhibition assay

Serological analysis was conducted using haemagglutination inhibition (HI) assays as previously described using either native virus for virus differentiation or ether/Tween-80 treated virus for diagnostic purposes (Daly et al., 1996). For virus differentiation the untreated viruses were assayed using ferret sera pre-treated with heat and periodate, using 1% chicken erythrocytes as previously described. 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 and A/eq/South-Africa/4/03.

2.4. Viral RNA isolation, RT-PCR and sequencing

Viral RNA was isolated from nasopharyngeal swabs or allantoic fluid using the QIAamp Viral RNA mini kit (Qiagen) according to the manufacturer's instructions. Genes of interest were amplified using the RobusT I RT-PCR kit (Finnzymes) with 2 μl of template RNA and 0.1 U/ml RNAsin (Promega) using the gene specific primers H3HA1/1 (5′-AGCAAAAGCAGGGGATATTTC) and H3HA1/2 (5′-GCTATTGCTCCAAAGATTC) for HA1 and primers NS1/EcoI (5′-CGGAATTCAGCAAAAGCAGGGTGACAAA) and NS1/Xba2 (5′-GCTCTAGAAGTAGAAACAAGGGTGTTTTTTATC) for NS1 at a final concentration of 0.2 μM. Samples were incubated 30 min at 48 °C and 2 min at 94 °C for the RT reaction, followed by 5 min at 94 °C, 1 min at 55 °C and 4 min at 72 °C for 30 cycles. PCR products were analysed on a 1% agarose gel stained with ethidium bromide and purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. PCR products were sequenced using ABI BigDye® Terminator v3.1 (Applied Biosystems) according to manufacturer's instructions on an ABI PRISM® 3100 Genetic Analyser (Applied Biosystems). Nucleotide sequences were visualised and edited using Seqman II version 5.03 (DNAstar Inc.).

2.5. Phylogenetic trees, multiple sequence alignments and amino acid mapping

To determine the relationship between the EIV isolates a phylogenetic tree was constructed. MODELTEST was used to determine the best evolutionary model for the data (http://hcv.lanl.gov/content/sequence/findmodel/findmodel.html) (Posada and Crandall, 1998). Phylogenetic trees were constructed using PAUP version 4.0 (Swofford, 1999) under the General Time Reversible substitution model, as determined by MODELTEST, with branch swapping by tree-bisection-reconnection. One hundred bootstrap replicates were conducted to assess the statistical support for the tree topology. Accession numbers of the virus strains in the trees are as follows (HA1, NS1): A/eq/Miami/63 (M29257, CY028840), A/eq/Fontainebleau/79 (CY032405, CY032409), A/eq/Newmarket/79 (D30677, AF001662), A/eq/Kentucky/2/81 (CY028820, CY028824), A/eq/Sussex/89 (X85090, FJ375212), A/eq/Rome/5/91 (D30684, AF001669), A/eq/Hong-Kong/92 (L27597, AF001670), A/eq/Lambourn/92 (X85087, AF001672), A/eq/Newmarket/2/93 (X85089, FJ375211), A/eq/Grobois/98 (AY328471, FJ195452), A/eq/Lincolnshire/1/02 (FJ195450, FJ195447), A/eq/Aboyne/05 (EF541442, FJ195444), A/eq/Kentucky/1/92 (CY030149, CY030153), A/eq/Alaska/1/91 (CY030157, AF001667), A/eq/Kentucky/98 (AF197241, FJ195446), A/eq/Newmarket/1/93 (X85088, FJ375210), A/eq/Kentucky/97 (AF197249, FJ195445), A/eq/South-Africa/4/03 (Prof. Alan Guthrie, Personal Communication), A/eq/Kentucky/9/04 (FJ195451, FJ195448), A/eq/Ohio/03 (DQ124192, DQ124186), A/eq/Wisconsin/1/03 (DQ222913, DQ222917), A/eq/Newmarket/5/03 (FJ375213, FJ375209), A/eq/Essex/1/05 (EF541439, FJ195449). In the HA1 tree (Fig. 1a) A/eq/Southampton/1/07 was representative of A/eq/Southampton/2/07 and A/eq/Solihull/2/07, A/eq/Cheshire/3/07 was representative of A/eq/Horsham/07, A/eq/Berkshire/07 and A/eq/Maidstone/2/07, A/eq/Richmond/1/07 was representative of A/eq/Maidstone/1/07, A/eq/Solihull/1/07 and A/eq/Strathaven/07. In the NS1 tree (Fig. 1b) A/eq/Richmond/1/07 was representative of A/eq/Strathaven/07, A/eq/Maidstone/1/07, A/eq/Richmond/2/07, A/eq/Solihull/1/07 and A/eq/Solihull/2/07, A/eq/Southampton/1/07 was representative of A/eq/Southampton/2/07.

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

3. Results

3.1. Isolation of EIV from the UK

A total of 28 NP-ELISA positive nasopharyngeal swabs were received in 2006 and 2007 from the UK, and from 20 of these, virus was recovered following growth in eggs (Table 1). The following outlines the clinical and vaccination histories of the affected animals.

In 2006, A/eq/Cheshire/1/06 was isolated from a large mixed yard of horses of different ages. At least 10 animals were infected and showed typical equine influenza (EI) clinical signs, two of which were recently vaccinated. A/eq/Southampton/1/06 was isolated from one of four infected horses that had uncertain vaccination histories. The NP-ELISA positive horse from Stowmarket in the UK had travelled from Ireland 2 days prior to showing typical clinical signs of EI and was unvaccinated. A/eq/Lanark/1/06 was isolated from a horse that was imported from Poland 3 days before showing clinical signs of infection. It had an uncertain vaccination history. A/eq/Lincolnshire/1/06 was isolated from a British spotted pony that had been in contact with a group of recently imported Friesian horses. Clinical signs were typical and included cough, nasal discharge and reduced appetite. The pony was unvaccinated.

In 2007, A/eq/Horsham/1/07 was isolated from an infected horse on a large dressage yard. Clinically the horse had pyrexia of 41.4 °C, nasal discharge and dyspnoea. The horse had an uncertain vaccination history but had recently been imported from Holland. The positive HI test

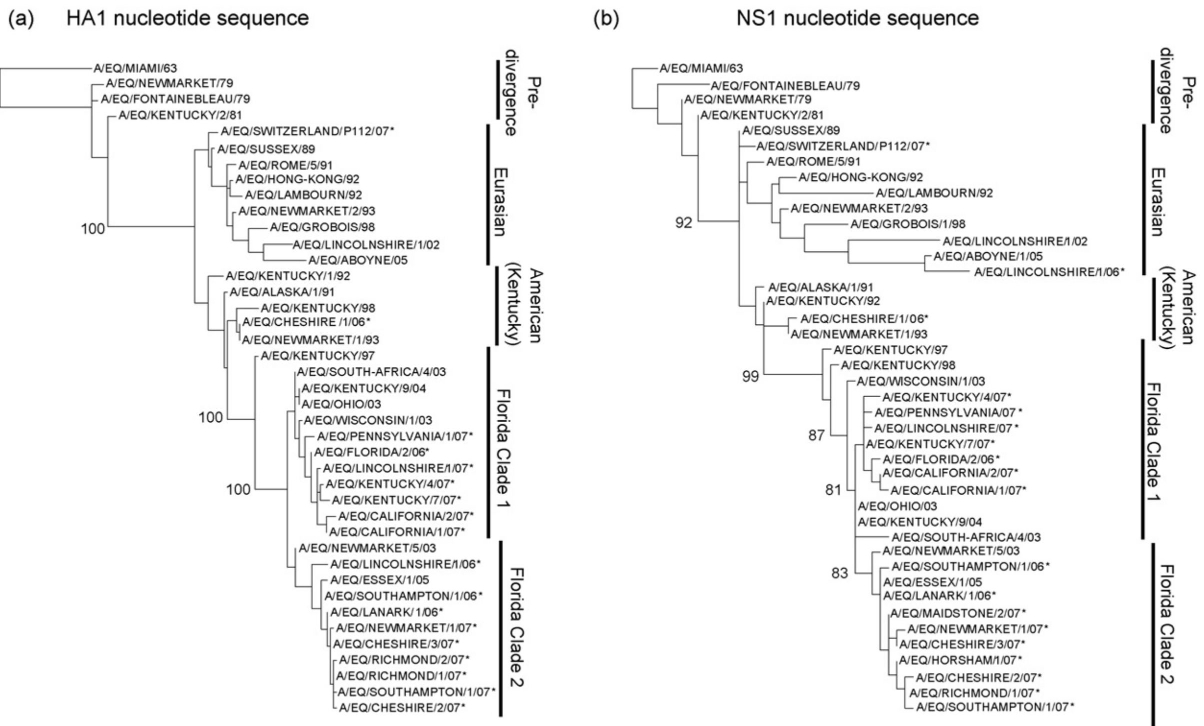

Fig. 1. Phylogenetic analyses of the HA1 (a) and NS1 (b) nucleotide sequences encoded by EIV, subtype H3N8. Maximum likelihood trees were created using PAUP version 4.0. Bootstrap values obtained after 100 replicates are shown at the major nodes. Phylogenetic groups are shown by continuous bars on the right and are labeled as appropriate. Accession numbers for the genes reported in this manuscript are listed in Table 1 and representative sequences are shown on the trees by an asterisk (*) as described in Section 2. Reference strains used in the trees are listed in Section 2.

from Stoke-on-Trent was from a non-vaccinated 6-year-old gelding which presented with a marked pyrexia of 40.6 °C, anorexia, profuse serous nasal discharge, frequent harsh dry cough, lymphadenopathy and mild ataxia. The onset of clinical signs was reported 4–5 days earlier. Four foci of EIV infection in the Midlands, Kent and Hampshire were identified from the end of May to the end of June 2007 (Solihull, Maidstone, Southampton). Nasopharyngeal swabs were taken from horses showing typical clinical signs of pyrexia with rectal temperatures up to 41 °C, profuse and frequently mucopurulent nasal discharge and frequent, harsh, dry cough with substantially increased respiratory rates. In all four outbreaks the index cases were animals that were recently imported into the country, having been bought at a horse sale in County Kilkenny in the Republic of Ireland on the 26th May. All these horses were reported to either be unvaccinated or of unknown vaccination history. There was one case of vaccine breakdown where a 6-year-old Irish sports horse resident at the Solihull yard showed clinical signs having previously been vaccinated with Equilis Prequenza TE^{TM} (Intervet) on 13th April 2007 and 8th May 2007. A/eq/Strathaven/1/07 was isolated from a 13-year-old Shetland pony with typical EI clinical signs of inappetence and ataxia. Nine further ponies were reported to be affected. This pony was on premises where horses had returned back sick from the Royal Highland Show near Edinburgh. There were no mandatory vaccination requirements for horses attending the show and many of them were consequently not vaccinated. The horse from Hawick which tested positive by NP-ELISA was shipped over from Irish horse sales around 14th August 2007. It was vaccinated with Equip FT^{TM} (Schering Plough) on 10th April 2006 and 8th May 2006 and then with Prevac Pro^{TM} (Intervet) on 11th November 2006. Clinical signs of inappetence, pyrexia, cough, mucopurulent nasal discharge, swollen lymph nodes and lethargy were observed. The positive NP-ELISA from Stowmarket was an 8-year-old pony mare, last vaccinated with Prevac T Pro^{TM} (Intervet) 12th July 2005 showed clinical signs consisted of pyrexia of 39 °C with unilateral nasal discharge and lethargy. A/eq/Lincolnshire/1/07 was isolated from a horse imported into the UK from Spain. The lorry had been elsewhere in Europe prior to this and there were no reported cases of influenza in horses on the yard in Spain from where it came from. It was showing typical clinical signs of EI on arrival. Two in contact animals subsequently had clinical signs of EI and one was identified as NP ELISA positive. Three separate viruses were isolated from 3 different sites in Cheshire. The first virus was isolated from a 2.5-year-old gelding that presented with a very mild pyrexia of 38.4 °C with slight mucopurulent nasal discharge. Several in contact horses were coughing and had mucopurulent nasal discharges with mild pyrexia. At the second site, 9 horses were affected and there were 47 in contact horses. Vaccinated animals appeared healthy throughout. At the third site a disease outbreak occurred on a large livery yard that was restricted to unvaccinated young horses. A/eq/Berkshire/1/07 was recovered from a

vaccinated horse newly imported from Holland although the exact vaccine was unknown. Two national hunt horses from Lambourn tested positive by NP-ELISA, both were fully vaccinated. The first horse received ProteqFlu™ (Merial) for the last 3 vaccinations and was last boosted August 1st 2007 (prior to this was 6th January 2007). The second horse received Equilis Prequenza TE™ (Intervet) as the last vaccination on 28th June 2007, ProteqFlu™ (Merial) before that on January 6th 2007 and Duvaxyn IE-plus™ (Fort Dodge) before that on 13th June 2006. A/eq/Newmarket/1/07 was isolated from an infected horse from one of three affected training yards. The animal had shown only mild signs and had been most recently vaccinated with ProteqFlu™ (Merial).

3.2. Genetic characterisation

3.2.1. HA1

The HA1 (1009 bp) from each virus isolate was sequenced and accession numbers are listed in Table 1. EIV isolates were grouped into 5 well- supported clades consisting of the Pre-divergent, Eurasian and American lineages with the Florida sublineage clades 1 and 2 (Fig. 1a). Phylogenetic analysis grouped 18 of the 20 isolates from the UK within the Florida sublineage clade 2. The isolate designated A/eq/Lincolnshire/1/07 was unique among the UK isolates as it clustered with the Florida clade 1 sublineage viruses. The isolate designated A/eq/Cheshire/1/06 grouped with the American (Kentucky) lineage viruses. The isolate from Switzerland was different from those isolated in the UK over the same time period as it belonged to the Eurasian lineage and clustered alongside the older strain A/eq/Sussex/89 (Fig. 1a). The HA1 of the North American virus isolates characterised here all clustered in the Florida sublineage clade 1, typical of the viruses isolated in or originating from North America (Lai et al., 2001) (Fig. 1a).

3.2.2. NS1

The NS1 (692 bp) coding sequence of each virus isolate was determined and accession numbers are listed in Table 1. The topology of the maximum-likelihood tree showed four clades corresponding to the Pre-divergent, American, Eurasian and Florida lineage viruses similar to those seen with the HA1 coding sequences. There was evidence of further differentiation into two different clades in the Florida sublineage cluster, as seen with HA1, the bootstrap value for this division in NS1 was 81% compared to 100% for HA1 (Fig. 1b). However, A/eq/Lincolnshire/1/06, which was within the Florida sublineage when analysing the HA1 coding sequence, grouped with the Eurasian lineage when analysing the NS1 coding sequence, and A/eq/Kentucky/98, which was American lineage by HA1 phylogeny was more similar to the Florida clade 1 viruses by NS1 phylogeny (Fig. 1b). The two isolates that were unusual according to their HA1 sequences, A/eq/Cheshire/1/06 and A/eq/Switzerland/P112/07, grouped as their HA1 sequences did, with the American lineage and Eurasian lineage NS1 sequences respectively. Both phylogenetic trees in Fig. 1 contain only unique sequences and any isolates with identical sequences were omitted as described in Section 2.

3.3. Antigenic analysis of EIV isolates

The antigenic properties of the EIV isolates were evaluated using ferret antisera raised against a panel of 8 AHT reference strains (Table 2). The isolate A/eq/Berkshire/1/07 was not characterised by HI assay as it only grew to a low titre in eggs after repeated passage. The American lineage virus and the Florida sublineage clades 1 and 2 viruses isolated in the UK and North America exhibited low reactivity to the antisera raised against Eurasian lineage isolates with the exception of A/eq/Pennsylvania/1/07, for which the highest titre obtained was against the A/eq/Benelux/03 antisera, although titres were generally low across the entire reference panel. The 17 Florida sublineage clade 2 viruses isolated from the UK and tested by HI all showed similar patterns of reactivity with the antisera, with 16 of the 17 strains showing an increase of between 2- and 4-fold in reactivity against A/eq/Newmarket/5/03 when compared to A/eq/South-Africa/4/03, and one strain showing a greater than 4-fold increase. Conversely 5 of the 7 Florida clade 1 viruses, one of which was isolated in the UK (A/eq/Lincolnshire/1/07) and the rest from North America, reacted 4-fold or more higher against A/eq/South-Africa/4/03 when compared to A/eq/Newmarket/5/03. The 2 remaining viruses, namely A/eq/California/2/07 and A/eq/Pennsylvania/1/07 both reacted higher to the A/eq/South-Africa/4/03 but only to a level of 2-fold or less respectively. Another trend between the clades 1 and 2 Florida sublineage viruses was their reactivity with the antiserum raised against A/eq/Kentucky/97, an early member of the Florida sublineage. The majority of clade 1 viruses raised relatively low titres ($\leq$32) against A/eq/Kentucky/97, with the exception of A/eq/Kentucky/4/07 and A/eq/Kentucky/7/07, whereas the clade 2 viruses raised higher titres of between 91 and 512. In contrast to the Florida sublineage clade 2 viruses isolated in the UK, A/eq/Cheshire/1/06, classified as an American lineage virus, had relatively low reactivity with the A/eq/Kentucky/97 antiserum and is more similar to those titres seen with the Florida clade 1 viruses from North America. It also reacted weakly across the panel when compared to A/eq/Newmarket/1/93, even though the predicted HA1 amino acid sequence is identical.

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

3.4. Amino acid alignments

In order to identify which amino acids may play a role in the separation into different phylogenetic clades, amino acid sequence alignments of the HA1 and NS1 coding sequences were constructed with representative isolates aligned against A/eq/Newmarket/5/03. Any HA1 changes are summarised in Table 3, NS1 changes are highlighted in Table 4. The numbering of the amino acid positions of the HA1 sequences starts with the serine residue immediately downstream of the predicted signal peptide cleavage site as 1. This was consistent with the numbering used for the

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 (FC1)	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/Lincolnshire/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–A/eq/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.

H3 HA structure (Ha et al., 2003). Negative numbers represent the predicted signal sequence. All the recent isolates had the substitutions at amino acids 190 and 193 when compared to A/eq/Newmarket/1/93, predicted to be within antigenic site B and first observed in A/eq/Newmarket/5/03 with the exception of A/eq/Cheshire/1/06 that was more similar to A/eq/Newmarket/1/93. The viruses belonging to the Florida sublineage clade 1 (North American isolates A/eq/Florida/2/06, A/eq/California/2/07, A/eq/Kentucky/4/07, A/eq/Kentucky/7/07, A/eq/Pennsylvania/1/07 and the UK isolate A/eq/Lincolnshire/1/07) all had very similar HA1 sequences in which the majority differ from American and Eurasian lineage viruses at residues −16, 62, 78, 104, 138 and 159. Between A/eq/South-Africa/4/03 and the latest Florida sublineage clade 1 isolates there were 10 different amino acid substitutions, one of which was in the signal sequence. The isolates A/eq/California/2/07 and A/eq/Kentucky/7/07 both contained 6 of these amino acid substitutions. A/eq/Pennsylvania/1/07 and A/eq/Ibaraki/07 shared the substitutions at residues V78A and N159S but not the changes at residues R62K, D104N or A138S with the other clade 1 isolates. They also had the additional unique changes at residues P162S and Q189K and when compared to A/eq/Newmarket/5/03. The Florida sublineage clade 2 viruses isolated in the UK including, A/eq/Lincolnshire/1/06, A/eq/Richmond/1/07 and A/eq/Newmarket/1/07, had a unique two amino acid insertion in the predicted signal peptide at residues −11

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	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	*	.	.	.	*
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.

and −10, including one aromatic residue, combined with a leucine to phenylalanine substitution at −9, to generate a 'FIF' or 'FNF' motif. The predicted HA1 sequences of A/eq/Newmarket/1/93 and A/eq/Cheshire/1/06 were identical, with substitutions at residues −2, 5, 30, 48, 58, 190, 193, 272 and 289 when compared to A/eq/Newmarket/5/03. The Eurasian lineage viruses A/eq/Sussex/89 and A/eq/Switzerland/P112/07 shared many of the amino acid changes listed above for the American lineage viruses, but also have some specific changes at T163I, K261R and I276T.

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

4. Discussion

EIV surveillance was conducted in order to identify and characterise currently circulating and new emerging virus strains to provide data for vaccine strain selection. The surveillance data for the UK and Europe was consistent with the previous observations that there was a predominance of Florida sublineage clade 2 viruses in circulation (Newton et al., 2006; Damiani et al., 2007). The index cases of the outbreaks that occurred in June 2007 in the UK were animals that had been recently imported into England, having been bought at a horse sale in County Kilkenny in the Republic of Ireland. It was therefore likely to be at least partially representative of the viruses circulating in the Republic of Ireland at that time (Newton et al., 2007). Florida sublineage clade 1 viruses were isolated in North America. Interestingly outbreaks of disease often follow horse movement and introduction into new environment. This may be due to stress associated with travelling making the animal more susceptible to infection.

The topology of the maximum-likelihood tree of the HA1 data was similar to that previously described (Fig. 1a) (Daly et al., 1996; Lai et al., 2001) and showed that viruses from the two Florida sublineage clades 1 and 2 continued to co-circulate in 2006 and 2007. Amino acid alignment of 2003 Florida clades 1 and 2 virus isolates suggested that the changes V78A and N159S were consistent between the clades (Table 3). They also identified an insertion within the signal peptide that created a 'FIF' motif at position −11 to −9 in the Florida sublineage clade 2 viruses isolated in

the UK. This insertion was first seen in isolates from 2004 and has been seen in all UK isolates belonging to clade 2 since then (e.g. A/eq/Wales/1/05, accession number EF541438). The authors note that the HA sequence of a virus isolated during the Mongolian outbreak in 2008 has been released to GenBank that indicates that this belongs to the Florida clade 2 sublineage (accession number AB436910). The amino acid alignment in Table 3 shows an isoleucine to an asparagine residue substitution at position −10 in A/eq/Newmarket/1/07, the last 2007 isolate described in this paper that changes the 'FIF' sequence to 'FNF'. This change was not seen in the Mongolian isolate suggesting this virus had a greater similarity to the older UK clade 2 isolates (data not shown).

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

As the UK and North America have two of the few active surveillance systems, it is not known which are the most widely circulating viruses globally and certain phylogenetic lineages may be over represented. Horse movement is a major contributing factor in transmitting EIV around the world, despite mandatory vaccination and quarantine procedures. Epidemiological and phylogenetic studies of EIV outbreaks have often found direct links with international horse movement such as in South Africa in 1986 (Guthrie et al., 1999), Hong Kong in 1992 (Lai et al., 1994) and Newmarket in 2003 (Newton et al., 2006). EIV was responsible for several large outbreaks of respiratory disease in 2007. In mid August 2007 an outbreak of EIV was detected in a partially vaccinated horse population in Japan (Yamanaka et al., 2008). Table 3 includes the partial sequence of A/eq/Ibaraki/07, a virus representative of the outbreak strain (Yamanaka et al., 2008). Horses were vaccinated with an inactivated vaccine containing A/eq/LaPlata/93 (American lineage), A/eq/Avesta/93 (Eurasian lineage) and A/eq/Newmarket/77 (H7N7). These strains, although dated, are not unlike the strains used in the vaccines available in the UK (Fig. 1). This raised the concern that currently available vaccines may also be vulnerable to breakdown when used to protect against this or a related virus, as seen in 2003 with the emergence of A/eq/Newmarket/5/03 which infected both vaccinated and unvaccinated equids in Europe. A/eq/Ibaraki/07 had highest sequence identity with A/eq/Pennsylvania/1/07, a Florida sublineage clade 1 virus with an HA1 sequence typical of those circulating in North America. However, the HI data presented in Table 2 shows that sera raised against currently used vaccine strains and those recommended by the OIE (OIE, 2008) raise relatively low titres to A/eq/Pennsylvania/1/07. It remains to be determined whether this virus represents a new clade within the evolution of EIV or whether representative strains are still circulating in the field. The HA1 amino acid alignments show substitutions P162S and Q189K in A/eq/Pennsylvania/1/07 which are not found in the other clade 1 viruses described in this report. A/eq/South-Africa/4/03 virus that reacts highly to antisera raised against A/eq/Kentucky/98 (Table 2) and to antisera raised against itself, only differs at these two amino acids within HA1, suggesting these two changes may be responsible for the differences in antigenicity observed between the 2 viruses. Residue 189 maps adjacent to the receptor-binding pocket on the structure of H3 and was exposed on the surface (Ha et al., 2003). The substitution P162S has been previously observed in some Pre-divergent strains such as A/eq/Tokyo/71 (accession number: M24720) but this is on a substantially different backbone sequence that only has 85.8% sequence identity within HA1 when compared to A/eq/Newmarket/5/03. The isolate A/eq/California/2/07 also had low reactivity to the antisera in Table 2. This strain contained 6 amino acid substitutions when compared to A/eq/South-Africa/4/03, of which R62K, T192K and K207E mapped at or near the surface of the HA1 molecule when aligned with the three dimensional structure of H3 HA (Ha et al., 2003) (data not shown). Later in the same month in 2007 there was an outbreak of EIV in Australia, this time mainly in an unvaccinated population after the virus escaped from a quarantine station into the general horse population. Subsequently the two outbreaks have been linked and a representative virus from the Australian outbreak (A/eq/Sydney2888-8/07) was almost identical to A/eq/Ibaraki/07 (Callinan, 2008; Yamanaka et al., 2008). The introduction of horse movement restrictions and horse free buffer zones in conjunction with vaccination using ProteqFlu^{TM} (Merial) which expressed both A/eq/Kentucky/94 and A/eq/Newmarket/2/93 HA, finally eradicated EIV from Australia.

Currently there are two recommended components to the vaccine, a Eurasian isolate and a Florida sublineage

clade 1 isolate (OIE bulletin, 2008). Many vaccine manufacturers have yet to update their vaccines as recommended by the OIE, although recently 2 manufacturers have done so with the inclusion of A/eq/Ohio/03 (ProteqFlu™, Merial and Calvenza™, Boeringher Ingelheim). As the antigenic distance increases between vaccine strains and those circulating, so does the risk of EIV infection in the field associated with vaccine breakdown. If both Florida sublineage virus clades persist and diverge in the field, vaccines may have to be updated to allow both clades to be represented in order to confer clade specific immunity.

Vaccine studies have shown that currently available vaccines reduce virus shedding and clinical signs when compared to control ponies when challenged with recent strains of EIV (Paillot et al., 2008; Bryant et al., 2008). However, in these studies, ponies were challenged two weeks post-vaccination under optimal experimental conditions more accurately modelling vaccination in the face of infection. Vaccine breakdown in the field did occur in Japan among racehorses previously vaccinated 3 months before the outbreak (Yamanaka et al., 2008). Other experiments in ponies have shown protection induced by non-adjuvanted vaccines containing virus from one lineage against challenge infection with heterologous virus is reduced when compared to challenge with the homologous virus, as in the case of vaccination with A/eq/Newmarket/2/93 (Eurasian) and subsequent challenge with A/eq/Newmarket/1/93 (American). Interestingly, this lack of protection occurs in one direction only in that vaccination with A/eq/Newmarket/1/93 does protect against challenge with A/eq/Newmarket/2/93 (Daly et al., 2004), suggesting the American lineage isolates may confer some protection against Eurasian isolates in the future.

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

In conclusion, 27 EIV strains from North America and Europe isolated in 2006–2007 were characterised in detail, in order to aid the vaccine strain selection process. Viruses belonging to the American (Kentucky) lineage, Eurasian lineage and Florida sublineage have been isolated with varying degrees of antigenic drift occurring from vaccine strains. The majority of isolates belonged to the Florida sublineage. Sequence divergence and antigenic differences support the further division of this group into 2 clades. To date, Florida clade 1 viruses have been isolated in North America with outbreaks in Japan and Australia likely to be due to horse movements. Clade 2 viruses continue to predominate in Europe. This has reinforced the importance of continued surveillance in the field in order to identify any new emerging threats to the equine industry.

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