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Using epidemics to map H3 equine influenza virus determinants of antigenicity

Alana Woodward, Adam S. Rash, Elizabeth Medcalf, Neil A. Bryant, Debra M. Elton *

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

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ABSTRACT

Equine influenza is a major cause of respiratory infections in horses and causes widespread epidemics, despite the availability of commercial vaccines. Antigenic drift within the haemagglutinin (HA) glycoprotein is thought to play a part in vaccination breakdown. Here, we carried out a detailed investigation of the 1989 UK outbreak, using reverse genetics and site-directed mutagenesis, to determine the individual contribution of amino acid substitutions within HA. Mutations at positions 159, 189 and 227 all altered antigenicity, as measured by haemagglutination-inhibition assays. We also compared HA sequences for epidemic and vaccine strains from four epidemics and found that at least 8 amino acid differences were present, affecting multiple antigenic sites. Substitutions within antigenic site B and at least one other were associated with each outbreak, we also identified changes in loop regions close to antigenic sites that have not previously been highlighted for human H3 influenza viruses.

Introduction

It is well known that mammalian influenza viruses undergo antigenic drift through acquisition of amino acid substitutions within the viral glycoproteins haemagglutinin (HA) and neuraminidase (NA), leading to the occurrence of disease epidemics. For this reason, vaccine strains to influenza viruses eventually become ineffective, unless they are updated regularly. The HA1 domain appears to be under the most selective pressure, consistent with its role in induction of neutralising antibodies (Nelson and Holmes, 2007). Substitutions in human H3 viruses have been associated with changes in charge, acquisition of glycosylation sites and also alteration of receptor binding avidity (Blackburne et al., 2008; Kobayashi and Suzuki, 2012; Lin et al., 2012). Changes in HA are often accompanied by substitutions in NA and it is believed that the activity of HA and NA should be balanced (Mitnaul et al., 2000; Kaverin et al., 1998; Baigent and McCauley, 2001). The rate of antigenic change differs between influenza viruses of different species: human H3N2 viruses appear to drift more rapidly than either swine H3N2 or equine H3N8 viruses, as assayed by haemagglutination inhibition (HI) with ferret antisera. This has been demonstrated by antigenic cartography, which indicated that human H3N2 viruses underwent multiple 'cluster jumps' between 1968 and 2003 (Smith et al., 2004) whereas equine and swine viruses evolved fewer antigenic clusters over a similar period of time (de Jong et al., 2007; Lewis et al., 2011).

Influenza virus of the H3N8 subtype was first reported in horses in 1963 and subsequently spread around the world, affecting the UK in 1965 (Rose, 1966). Early reports suggested that equine influenza virus (EIV) did not undergo antigenic drift (Burrows et al., 1981), however it is now clear that the virus acquires mutations in HA that lead to antigenic drift in much the same way as other influenza A viruses (Daniels et al., 1985; Oxburgh et al., 1993; Lewis et al., 2011). Multiple lineages and sub-lineages have evolved since 1963, including the divergence of the Eurasian and American viruses of the late 1980s (Daly et al., 1996), followed by further division of the American lineage into the Kentucky, Argentinian and Florida clade 1 and 2 (FC1, FC2) sublineages (Lai et al., 2001; Bryant et al., 2009). Following the initial pandemic wave in 1965, there have been three further country-wide outbreaks in the UK in 1979, 1989 and 2003. As a result of the 1979 outbreak, which caused substantial disruption to the racing industry, mandatory vaccination was introduced for racing Thoroughbreds in the UK. Other competitive bodies now require vaccination for competition and rules are laid down by the British Horseracing Authority and Federation Equestra Internationale (FEI).

In 1989 the UK epidemic affected recently vaccinated and unvaccinated animals alike, indicating that the causative strain had undergone significant antigenic drift from the existing vaccine strains (Livesay et al., 1993; Binns et al., 1993). A similar outbreak occurred in Hong Kong in 1992, also affecting vaccinated animals (Powell et al., 1995). The vaccines in use at the time included the EIV prototype H3N8 strain A/equine/Miami/63, but also more recent pre-divergence strains from

* Corresponding author. Fax: +44 1638 555643.
E-mail addresses: alana.woodward@aht.org.uk (A. Woodward), adam.rash@aht.org.uk (A.S. Rash), elizabeth.medcalf@aht.org.uk (E. Medcalf), neil.bryant@aht.org.uk (N.A. Bryant), debra.elton@aht.org.uk (D.M. Elton).

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1979 to 1981. In both the 1989 UK outbreak and the Hong Kong outbreak the viruses belonged to the Eurasian sub-lineage of EIV (Lai et al., 1994, 2001). Following the 1989 outbreak in the UK, a surveillance programme was established to monitor antigenic drift in EIV and make recommendations for suitable strains for vaccine use.

In 2003, a large-scale outbreak started in Newmarket, affecting racing Thoroughbreds despite recent vaccination (Newton et al., 2006). Again, this was suggestive of antigenic drift, although the use of simple killed vaccines was also implicated. Vaccines in use at the time contained Eurasian and American (Kentucky) strains from the 1990s. A further example of large-scale vaccination breakdown occurred in Japan in 2007, in which vaccines contained Eurasian and American (Argentinian) strains from 1993 and the outbreak virus belonged to FC1 (Ito et al., 2008). A consistent feature of these large scale outbreaks was that commercial vaccines all contained strains that were at least 10 years older than the outbreak strain.

Antigenic characterisation of influenza viruses for the purpose of vaccine strain selection has been carried out using haemagglutination-inhibition (HI) assays for human and equine influenza viruses for many years (WHO, 2002). Ferret antisera distinguish readily between EIV strains, whereas equine antisera are typically more cross-reactive (Hinshaw et al.,1983; Burrows and Denyer, 1982). Current criteria used for the selection of EIV vaccine strains also include comparison of amino acid sequences for HA1. More than four amino acid changes in two or more antigenic sites, based on those mapped for human H3N2 viruses and/or an 8-fold or greater difference in HI titre between vaccine and circulating virus strains are considered significant (Wiley and Skehel, 1987; Wilson and Cox, 1990). However, the equine H3 molecule remains poorly characterised in this respect and antigenic sites are typically extrapolated from human H3 viruses (Barbic et al., 2009; Ito et al., 2008). The five antigenic sites of human H3 are thought to represent antibody-binding sites and were originally mapped on the basis of sequence variation amongst field strains and generation of escape mutants, selected by passaging viruses in the presence of monoclonal antibodies or neutralising human antisera (reviewed by Wiley and Skehel, 1987; Wilson and Cox, 1990). To date, these methods have not been applied consistently to EIV.

There is no legal requirement for EIV vaccines to be updated and the process from recommendation of new strains by the OIE to the appearance of updated commercial vaccines on the market typically takes several years. It is therefore important that reliable predictive methods are developed so that suitable recommendations can be made in a timely fashion. One area of importance is mapping the regions of EIV H3 that are important for antigenicity, rather than relying on those mapped for human H3 viruses. In this context, we undertook a detailed antigenic comparison of vaccine and outbreak strains from the 1989 UK outbreak. Reverse genetics and site directed mutagenesis were applied to determine the effect of individual amino acid differences between the outbreak and vaccine strains, with the aim of adding to current knowledge of the significant amino acid changes for equine H3. The study was extended by comparison of HA sequences from outbreak and vaccine strains associated with other known epidemics of equine influenza, using data from 1979, 1989, 2003 and 2007.

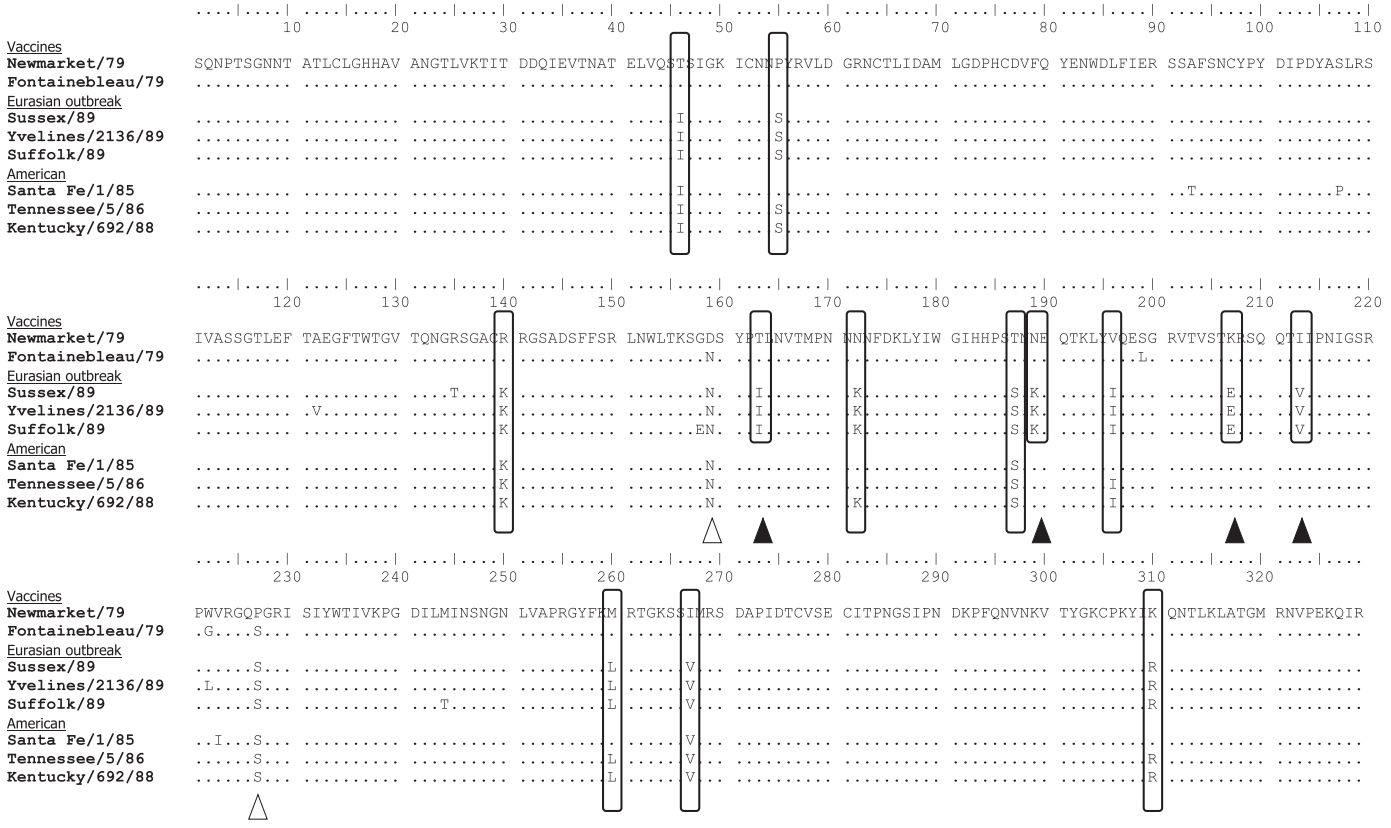

Fig. 1. Comparison of vaccine and outbreak EIV strains: 1989 UK outbreak. Derived amino acid sequences for HA1 were aligned for two vaccine strains and three isolates from the 1989 outbreak in Europe, three contemporary strains from the American (Kentucky) sub-lineage are included for reference. Amino acid substitutions between the 1979 vaccine strains and 1989 field strains are outlined in black. Substitutions unique to the Eurasian outbreak strains are indicated by black triangles, two further differences restricted to the Newmarket/79 vaccine strain are shown by open triangles. Residues are numbered from 1 to 329, starting with serine at the start of the predicted mature polypeptide for HA1. Genbank accession numbers are: Newmarket/79 – KJ643908, Fontainebleau/79 – KJ643904, Sussex/89 – KJ643906, Yvelines/2136/89 – BAA33940, Suffolk/89 – KJ643907, Santa Fe/1/85 – ACD85286, Tennessee/5/86 – ACA24656 and Kentucky/692/88 – ACA24579. Source of data and passage history are provided in Table S1.

Results

Comparison of HA1 sequences from 1989 outbreak isolates and vaccine strains

To identify conserved mutations between the 1989 outbreak strains and vaccine strains in use at the time, two representative vaccine strains and three outbreak strains from AHT archives were sequenced and derived amino acid sequences compared (Fig. 1). The vaccine strains were A/equine/Newmarket/79 (Newmarket/79) and A/equine/Fontainebleau/79 (Fontainebleau/79), which belong to the Pre-divergence sub-lineage of EIV; outbreak strains were A/equine/Sussex/89 (Sussex/89), A/equine/Suffolk/89 (Suffolk/89) and A/equine/Yvelines/89 (Yvelines/89) of the Eurasian sub-lineage. Contemporary viruses from the 1980s that belonged to the Kentucky sub-lineage (Lai et al., 2001) were included in the alignment for comparison (Fig. 1). The latter strains did not cause mass vaccine breakdown in Europe, unlike those belonging to the Eurasian sub-lineage. Comparison of the 1989 outbreak strains against the vaccine strains identified 13 amino acid substitutions that were common to all three 1989 outbreak strains, distributed throughout the HA1 sequence (Fig. 1). Comparison of these changes with EIV HA1 sequences available on Genbank to date indicated that they were common to all of the strains belonging to the Eurasian sub-lineage, isolated from 1989 onwards (data not shown). Of these substitutions, four were unique to the Eurasian lineage [T163I, N189K, K207E and I213V] and three involved changes in charge: N172K, N189K, K207E. A further two non-conservative changes were unique to the Newmarket/79 strain comparison: D159N and P227S whereas the Fontainebleau/79 vaccine had the additional differences S199L and W222G. Virus isolates belonging to the Kentucky (American) sub-lineage differed from the vaccine strains by up to 9 amino acids, depending on the isolate, but only N172K involved a change of charge (Fig. 1).

The locations of individual substitutions between vaccine and outbreak strains were mapped onto the H3 HA using the A/duck/Ukraine/63 structure available in PDB [1MLQ, Ha et al. (2003)] and are shown in the vertical and top views in Fig. 2(A) and (B). This was the closest structure available to the prototype H3N8 equine influenza virus; the mature HA without the signal sequence has the same number of amino acids as equine H3. Multiple amino acid differences were located to the top of the HA trimer, forming a striking ring of substitutions (Fig. 2B). Substitutions also occurred at other sites within the globular head, both on the surface and buried within the structure (Fig. 2A).

Antigenic analysis of parental wild type strains from 1989

To select a suitable prototype strain for further analysis, antigenic characterisation was carried out on low passage isolates from the 1989 outbreak. As described above, these strains belong to the Eurasian sub-lineage of EIV. The vaccine strains from 1979, included in commercial vaccines at the time of the outbreak, were also included to assess the ability of ferret sera to distinguish between the outbreak and vaccine strains. Since the number of strains available for 1989 and 1979 was limited, further strains belonging

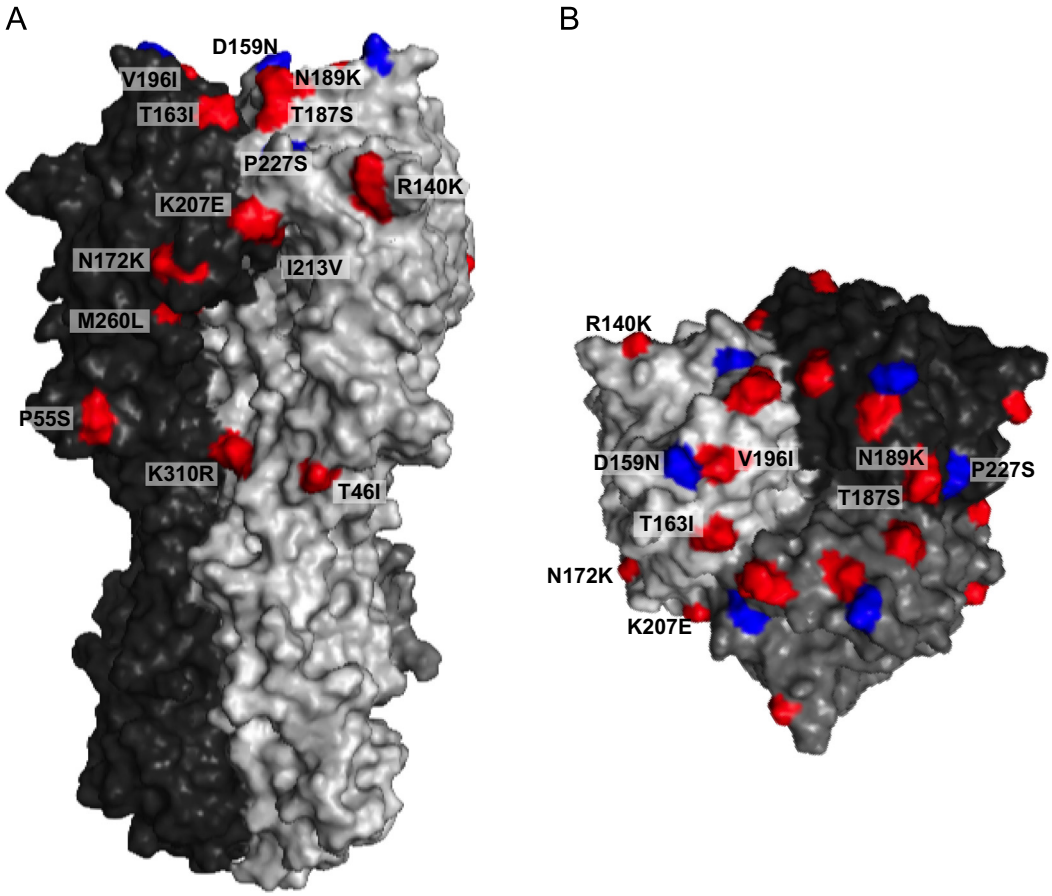

Fig. 2. HA structures: 1989 EIV outbreak. The locations of HA1 amino acid differences between vaccine and outbreak strains are indicated in red on the surface of the H3 HA structure for A/duck/Ukraine/1/63, protein database number PDB:1MLQ (Ha et al., 2003). Amino acid differences unique to Newmarket/79 are indicated in blue. Structures were examined using MacPyMOL and images collected for vertical (A) and top (B) views; the difference at HA1 residue 7 is not shown as the structure was solved for bromelain-cleaved HA and lacks the transmembrane domain (Ha et al., 2003).

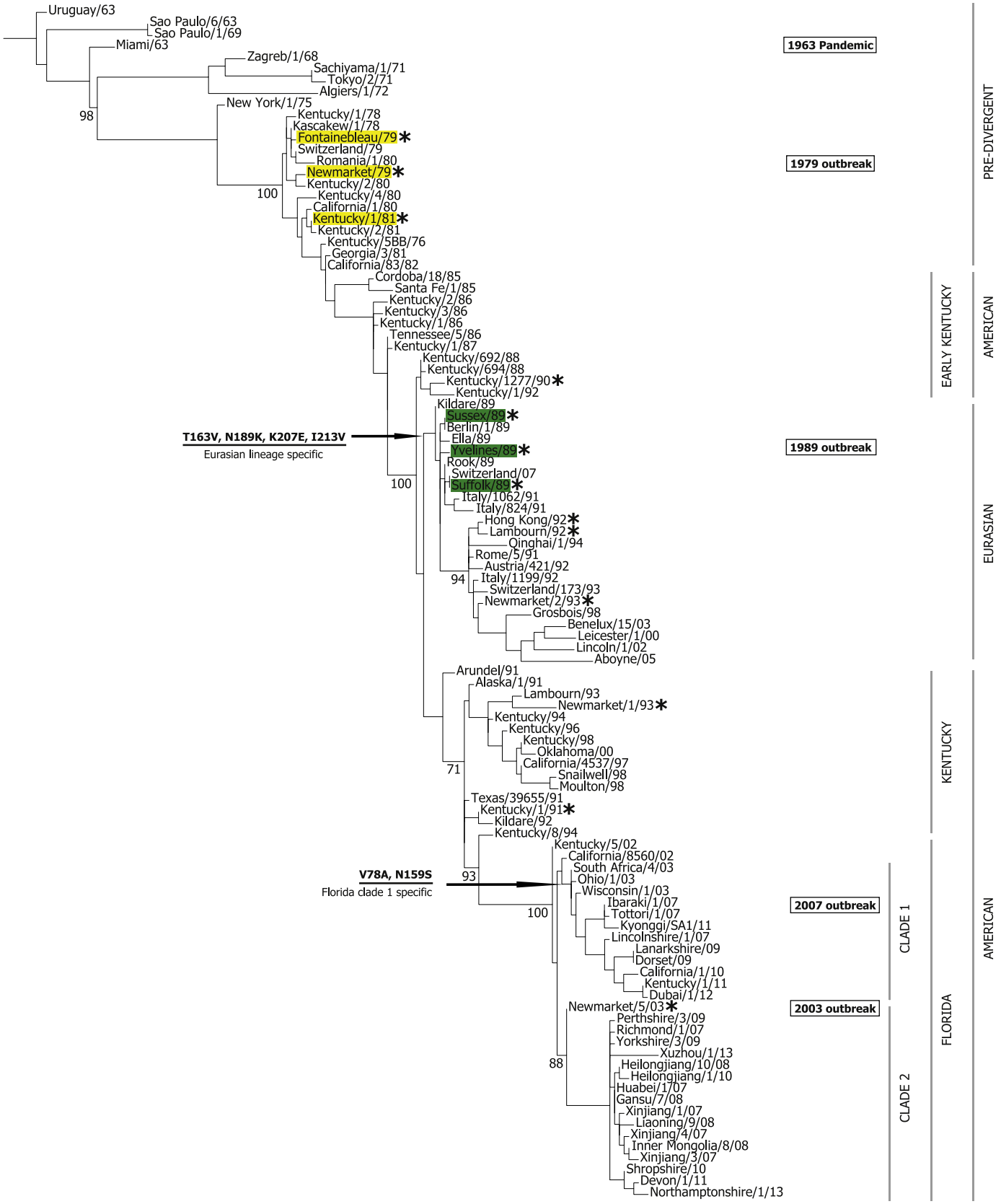

Fig. 3. HA1 Phylogenetic tree showing vaccine and epidemic strains. Maximum-likelihood tree based on the nucleotide sequence encoding HA1 for 103 representative EIV strains from major lineages and sub-lineages isolated between 1963 and 2013. Lineages, sub-lineages and major clades are indicated by bars to the right, large scale outbreaks are indicated in boxes and strains used in this manuscript are marked by asterisks. Vaccine strains are highlighted in yellow and outbreak strains are highlighted in green. Bootstrap values obtained from 100 replicates are indicated at major nodes, relevant amino acid substitutions are also shown at key nodes.

Table 1
Antigenic characterisation of parental EIV strains by HI assay. Ferret and equine antisera were raised following experimental infection in animals, unless indicated. Homologous titres for each virus are indicated in bold.

Lineage	Ferret antisera									Equine antisera			
	Pre-div.			Eurasian				American/Kentucky		Pre-div.	Eurasian		American/ Kentucky
	Newmarket/ 79	**Fontainebleau/ 79**	**Kentucky/ 81**	**Suffolk/ 89**	**Sussex/ 89**	**Yvelines/ 89**	**Lambourn/ 92**	**Hong Kong/ 92**	**Kentucky/ 90**	**Kentucky/ 91**	**Newmarket/ 79[a]**	**Sussex/ 89**	**Hong Kong/ 92**	**Kentucky/91**
Newmarket/79	**128**	32	< 8	< 8	< 8	8	< 8	8	8	8	**8**	< 8	< 8	8
Fontainebleau/ 79	64	**128**	32	< 8	8	16	< 8	8	64	32	16	< 8	< 8	8
Kentucky/81	32	128	**64**	< 8	16	16	8/16	< 8	256	64	32	< 8	< 8	8
Suffolk/89	32	32	< 8	**8**	16	32/64	16	128	< 8	16	< 8	16	8	32
Sussex/89	32	128	32	32	**256**	256	256	64	128	512	< 8	**32**	8	32
Yvelines/89	128	128	32	32	256	**256**	128	128	256	512	< 8	16	8	64
Lambourn/92	128	128	< 8	32	256	256	**256**	128	64	128	32	32	32	128
Hong Kong/92	64	64	< 8	16	64	64	32	**256**	< 8	32	< 8	64	**128**	512
Kentucky/90	64	128	64	< 8	16	32	8	8	**256**	64	8	8	< 8	16
Kentucky/91	128	256	128	8	32	64	16	32	512/1024	**128**	16	16	16	**128**

[a] Serum collected after sequential vaccination with A/equine/Miami/63 and Newmarket/79.

to the pre-divergence, Eurasian and American/Kentucky sub-lineages and their homologous sera were also included for comparison. For clarity, all these strains are highlighted in the phylogenetic tree for HA1 in Fig. 3 and an amino acid alignment is provided in Supplementary data Fig. S1. HI assays were carried out using a panel of post-infection reference antisera, raised in ferrets (Table 1). A limited number of equine sera were also available from AHT archives, against Newmarket/79, Sussex/89, Hong Kong/92 and Kentucky/91. Homologous titres against matched virus and ferret sera ranged from 64 to 256, with the exception of Suffolk/89, which had a very low homologous titre of 8. The homologous titres against equine sera ranged from 8 (Newmarket/79) to 128 (Kentucky/91, Hong Kong/92). Of the three 1989 outbreak strains, Suffolk/89 antigen gave a low titre against most of the antisera tested, and the ferret serum was also poor, it was therefore not selected for further work. This pattern of reactivity is not uncommon for equine influenza H3N8 strains (data not shown) and is typical of so-called 'low avidity' strains. The remaining two outbreak strains, Yvelines/89 and Sussex/89 gave similar results; most titres were within a two-fold difference against all the sera tested (Table 1). Sussex/89 was selected as a UK example of the outbreak in Europe.

Interestingly, the HI data demonstrated a one-way pattern of cross reactivity for sera raised against viruses belonging to different lineages. Ferret sera raised against the early pre-divergence (1979–1981) and Kentucky strains (1990–1991) cross-reacted with most of the strains belonging to the Eurasian lineage (Table 1). In contrast, ferret sera raised against the more recent Eurasian viruses (1989–1992) recognised other viruses from the same group and, to a lesser extent, the Kentucky sublineage viruses, but showed very little cross-reactivity against the pre-divergence strains isolated 10 years or more earlier. The pre-divergence strains were recognised by sera raised against similar viruses, as expected, but also by sera against the Kentucky viruses (titres from 8 to 256). Newmarket/79 gave a 4–8 fold lower titre against these ferret sera than Fontainebleau/79, which may have been due to the additional substitutions at positions 159 and 227 seen in this virus (Fig. 1). The pre-divergence viruses were poorly recognised by sera raised against viruses belonging to the Eurasian lineage, with titres ranging from <8 to a maximum of 16. As the Eurasian viruses were recognised by sera from all three lineages shown in Table 1, these data demonstrate clearly that older sera did not distinguish well between different strains and that up to date homologous sera were required to show differences in titre of up to 32-fold between pre-divergence and outbreak strains. The equine sera generally gave lower titres than the ferret sera, making the data more difficult to interpret. Equine sera against the Eurasian and Kentucky viruses gave a similar overall pattern, with both Eurasian sera giving titres of <8 against the pre-divergence vaccine strains. The serum against Newmarket/79 gave low titres against all the virus antigens tested; it failed to inhibit four of the five Eurasian strains. Of all the equine sera tested, Kentucky/91 gave the highest titres against most antigens but consistently gave a titre of only 8 to the three pre-divergence strains, a difference of 4–8 fold when compared against the 1989 Eurasian viruses. Taken together, the HI data indicated that antigenic differences were clear between the pre-divergence and Eurasian lineages, with the greatest differences seen using the most recent ferret sera against the Eurasian lineage.

Generation of 7:1 reassortment viruses in an H3N8 background

To analyse the antigenic effect of individual amino acid substitutions, reverse genetics was used to first construct wild type viruses containing HA of the vaccine strain Newmarket/79 and outbreak strain Sussex/89 in an identical genetic background. Segment 4 of each strain, encoding HA, was successfully rescued as a '7:1 reassortant' in the A/equine/Kentucky/02 (Kentucky/02)

Table 2
Antigenic comparison of EIV point mutants against WT reassortants by HI assay. HI assays were carried out for WT 7:1 reassortants and virus mutants, in either the Newmarket/79 HA background (top) or the Sussex/89 HA background (bottom), against a panel of post-infection antisera raised against pre-divergent strains Newmarket/79 (New/79), Fontainebleau/79 (Fon/79); Eurasian strains Yvelines/89 (Yve/89), Sussex/89 (Sus/89), Hong Kong/92 (HK/92), Newmarket/2/93 (New/2/93); American strains belonging to the Kentucky sub-lineage: Newmarket/1/93 (New/1/93), Kentucky/97 (Ken/97) and Florida clade 2 (FC2): Newmarket/5/03 (New/5/03). Homologous titres for each serum against its matched parental virus are shown in bold, titres for each WT reassortant virus are highlighted in grey. Assays for each mutant were replicated a minimum of 3 and up to 8 times then GMT values calculated and compared against the value for the appropriate WT reassortant. All titres of less <8 were assigned a value of 1 in order to calculate GMT values. The fold difference in HI titre is shown compared to the appropriate WT virus for each point mutant, values of 4-fold or more higher are highlighted in red, those of 4-fold or more lower are highlighted in blue.

	Ferret Antisera								
	New/79 Pre	Fon/79 Pre	Yve/89 Eur	Sus/89 Eur	HK/92 Eur	New/2/93 Eur	New/1/93 KY	Ken/97 KY/FC2	New/5/03 FC2
Homologous titre	**128**	**128**	**256**	**256**	**256**	**512**	**256**	**128**	**256**
New/79 reassortants									
Wild-type GMT	279.17	49.35	1.00	1.00	1.83	1.00	1.00	16.00	8.00
T46I	2.59	1.83	2.00	1.68	3.67	1.68	2.00	1.41	2.38
P55S	0.92	1.83	2.00	1.00	2.59	1.68	4.00	1.68	2.38
R140K	1.09	1.09	1.68	1.00	3.08	1.00	1.00	1.41	1.41
D159N	0.92	4.36	2.83	1.68	0.55	1.00	90.51	11.31	2.38
T163I	0.39	0.55	1.00	1.00	0.55	1.00	1.00	0.50	0.13
N172K	0.92	1.30	2.00	1.00	0.95	1.00	1.00	2.00	1.32
T187S	0.73	1.30	1.00	1.00	0.55	1.00	1.00	1.41	1.00
N189K	0.77	1.03	32.00	32.00	8.72	20.16	1.00	0.79	0.13
V196I	0.92	1.30	1.00	1.00	0.92	1.00	1.00	1.00	1.00
T187S, N189K, V196I	0.46	0.92	32.00	64.00	2.18	22.63	1.00	2.00	0.13
K207E	0.55	1.30	1.00	1.00	0.92	1.00	1.00	1.19	1.19
I213V	0.92	1.30	1.00	1.00	0.92	1.00	1.00	1.19	1.19
P227S	1.54	3.08	13.45	1.00	10.37	1.68	5.66	2.00	3.36
M260L	0.92	1.30	1.00	1.00	0.92	1.00	1.00	1.68	1.19
I267V	1.30	1.30	1.00	1.00	0.92	1.00	1.00	1.41	1.00
K310R	0.65	1.30	1.00	1.00	1.54	1.00	1.00	1.68	1.68
Sus/89 reassortants									
Wild-type GMT	5.94	57.97	141.32	190.21	26.25	172.28	23.78	95.10	13.13
S55P	1.90	1.10	1.52	1.35	1.45	1.77	0.67	0.80	0.72
K140R	0.95	1.56	2.56	1.90	2.44	2.10	1.60	1.35	1.22
N159D	2.69	0.02	0.23	0.40	0.51	0.26	0.04	0.12	0.08
N159D, I163T	3.81	0.02	0.19	0.34	0.11	0.26	0.04	0.02	0.08
T163I	2.26	1.31	0.91	0.80	1.22	1.05	1.35	1.13	0.72
K172N	1.90	1.10	1.28	1.13	1.45	1.49	0.67	0.80	0.61
S187T	1.60	1.46	1.37	0.89	0.92	0.74	1.35	0.89	0.70
K189N	0.17	1.10	0.01	0.01	0.04	0.01	1.35	1.13	0.86
I196V	1.35	1.10	0.91	0.67	1.22	1.25	0.95	0.67	0.22
E207K	3.20	1.10	1.08	1.13	1.22	1.49	0.95	0.80	0.61
V213I	0.67	1.10	1.81	1.35	1.22	1.87	0.67	1.35	0.61
S227P	0.17	0.05	0.16	0.12	0.11	0.19	0.12	0.24	0.22
L260M	1.35	1.31	1.52	1.13	2.05	1.77	1.13	1.13	0.72
V267I	2.69	1.10	1.52	1.13	2.44	2.10	1.35	0.95	1.22
R310K	1.90	0.93	0.91	0.67	1.45	1.05	0.80	0.80	0.72

reverse genetics system (Quinlivan et al., 2005). Fifteen individual substitutions were generated by site directed mutagenesis in a background of either Sussex/89 or Newmarket/79 HA, including the 13 changes between the vaccine and outbreak strains plus the additional two differences unique to Newmarket/79. Triple mutations, containing the three adjacent substitutions T187S, N189K and V196I, were made in the Newmarket/79 HA gene. These three substitutions were in close proximity and mapped on the top surface of HA (Fig. 2B). In the Sussex/89 background, an additional double mutant was constructed, combining N159D and I163T. These two changes again occurred at the top of the HA trimer (Fig. 2); Newmarket/79 vaccine strain had both changes compared with Sussex/89, whereas Fontainebleau/79 vaccine only differed at 163 (Fig. 1). Of the 32 resultant constructs, 31 were rescued successfully as 7:1 reassortants in Kentucky/02. Three independent attempts were made to rescue Sussex/89 I46T without success. The HA sequence of each virus was determined to confirm that there were no unwanted mutations prior to antigenic analysis and all viruses replicated to similar titres in eggs.

Antigenic characterisation of point mutants

Each virus mutant was characterised by HI assay against a panel of post-infection ferret antisera and compared against the appropriate recombinant WT virus (Table S2). Assays were carried out in duplicate and repeated at least three times, geometric mean titres (GMT) were then calculated. For ease of interpretation, fold differences were calculated between the WT values and each mutant and are provided in Table 2. Increases and decreases of four fold or more, generally accepted as significant for HI data, are highlighted in red and blue respectively. Ferret antisera from AHT archives were used against the two pre-divergence 1979 vaccine strains, four viruses belonging to the

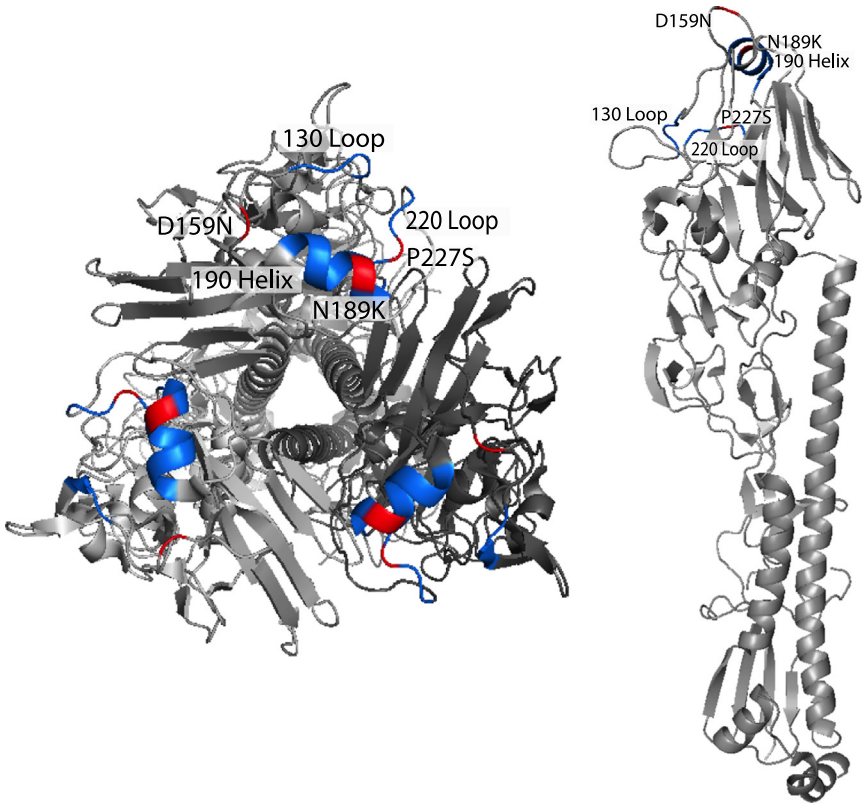

Fig. 4. Proximity of substitutions 159, 189 and 220 to the HA receptor-binding site. The locations of the three amino acid substitutions that caused the greatest antigenic effects are indicated in red on the H3 HA structure for A/duck/Ukraine/1/63, protein database number PDB:1MLQ (Ha et al., 2003). The 130 loop, 190 helix and 220 loop components of the receptor-binding site are indicated in blue. (A) trimeric structure, top view; (B) monomeric structure, side view.

Eurasian sub-lineage (Sussex/89, Yvelines/89, Hong Kong/92 and Newmarket/2/93), and three members of the American sub-lineage: one Kentucky (Newmarket/1/93), one with sequence and antigenic similarity to both Kentucky and FC2 (Kentucky/97) and one early FC2 isolate (Newmarket/5/03). The phylogenetic relationship between these strains is indicated in Fig. 3.

Antigenicity of Newmarket/79 point mutants

The Newmarket/79 series of mutant viruses had individual amino acid substitutions that switched the residue to that present in Sussex/89. The purpose was to determine whether any mutations either decreased recognition by antisera against the vaccine strains (Newmarket/79 and Fontainebleau/79) or increased recognition by antisera corresponding to the Eurasian outbreak strains (Sussex/89, Yvelines/89). The HI titres for the WT reassortant against the panel of ferret antisera (Table 2) indicated that only the antisera against Newmarket/79 and Fontainebleau/79 recognised the Newmarket/79 WT virus well, at GMT values of 279 and 49 respectively. The antisera raised against Eurasian strains of viruses did not recognise WT Newmarket/79, neither did the antiserum raised against the American lineage strain Newmarket/1/93, giving GMT titres below the level of sensitivity of the assay. However, low but measurable titres of 8 and 16 were obtained with antisera against the FC2 sub-lineage strain Newmarket/5/03 and the intermediate strain Kentucky/97.

HI assays were carried out for all of the point mutants and the fold differences between their titre and that of WT Newmarket/79 virus are shown in Table 2. The majority of the mutations had little antigenic effect and none reduced recognition by the Newmarket/79 or Fontainebleau/79 antisera by four fold or more, suggesting that a loss of antigenic recognition required more than one amino acid change. This was unsurprising, as Fontainebleau/79 serum also recognised Sussex/89 WT virus. However, three individual changes resulted in increased titres to one or more ferret serum. In more detail, changes at positions 46, 55, 140, 163, 172, 187, 196, 207, 213, 260, 267 and 310 had little or no effect (a difference of 2-fold or less in titre against most sera). Of the remaining mutations, D159N was the only change to have a pronounced effect on the reactivity to the vaccine sera, resulting in more than a 4-fold increase in titre to Fontainebleau/79 but having no effect on the titre against Newmarket/79. This was consistent with the difference in HA1 sequence at position 159 between the two vaccine strains (Fig. 1). Newmarket/79/D159N also showed a marked increase in titre to antisera against the Kentucky and Florida sub-lineage viruses Newmarket/1/93 and Kentucky/97, increasing the titre by 90-fold and 11-fold respectively. However, this mutation had little effect on antisera raised to the Eurasian viruses including Sussex/89 and Newmarket/2/93, despite all these viruses having asparagine (N) at position 159. This suggests that this particular mutation did not make a significant contribution to the antigenic differences between the 1979 and 1989 viruses but may be important for evolution of the American/Kentucky sub-lineage. The D159N substitution occurred on the top of the HA trimer, close to the receptor binding site but across the trimer interface (Figs. 2 and 4). In contrast, the most obvious increase in titre against the Eurasian antisera was observed for the single and triple mutants containing the substitution N189K. The difference was slightly more pronounced with the triple mutant T187S/N189K/V196I (Table 2). However, the single substitution at 189 was sufficient to cause a marked and specific increase in recognition by all four of the Eurasian antisera whilst having little effect on titres against American sera. This amino acid maps to the 190 loop of the receptor-binding site (Fig. 4). In contrast to the pattern seen for N189K, the P227S mutation showed more complex changes in antigenicity; this substitution specifically increased recognition by two of the Eurasian antisera, Yvelines/89 and Hong Kong/92, by more than 10 fold and

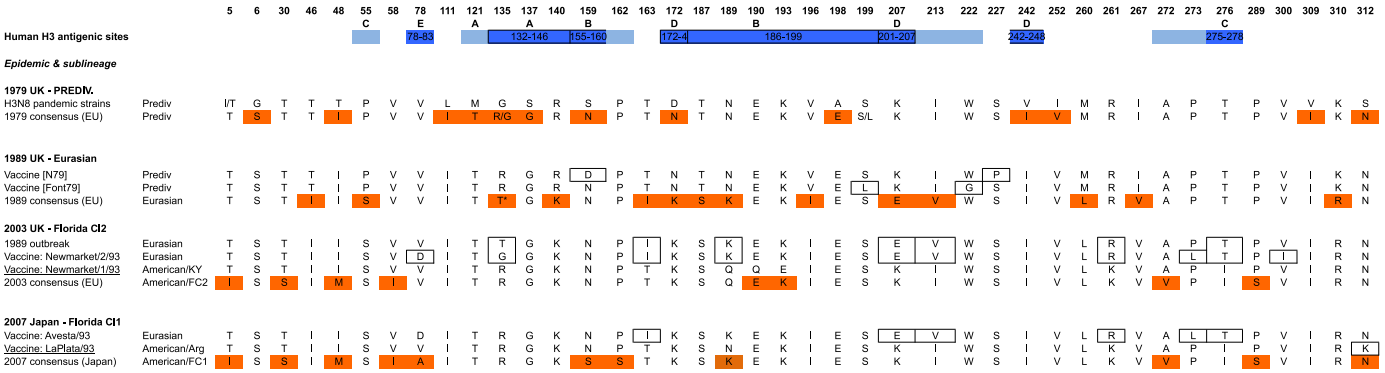

Fig. 5. HA1 substitutions associated with major EIV outbreaks. Amino acid substitutions between outbreak and relevant vaccine strains, or previous epidemic strains as appropriate, are highlighted in orange for major EIV outbreaks in 1979, 1989 (Binns et al., 1993), 2003 (Newton et al., 2006) and 2007 (Ito et al., 2008). Additional substitutions specific to only one vaccine strain (1979 outbreak) or the less related Eurasian vaccine strain (2003 and 2007 outbreaks) are outlined by boxes. The sub-lineage to which each outbreak belonged and the vaccine strains in use at the time are indicated: Prediv. – predivergence, FC2 – Florida clade 2, FC1 – Florida clade 1, Arg – Argentinian, KY – Kentucky. Consensus amino acids are shown for field strains from the early 1960s and for subsequent outbreaks, compiled from alignments of HA1 sequences available on Genbank (see Figs. S2–S5). The approximate positions of antigenic sites mapped for human H3 viruses are indicated in blue, from Wiley et al. (1981), Wiley and Skehel (1987) and Wilson and Cox (1990), the locations of substitutions close to antigenic sites or within the same loop regions are indicated in pale blue.

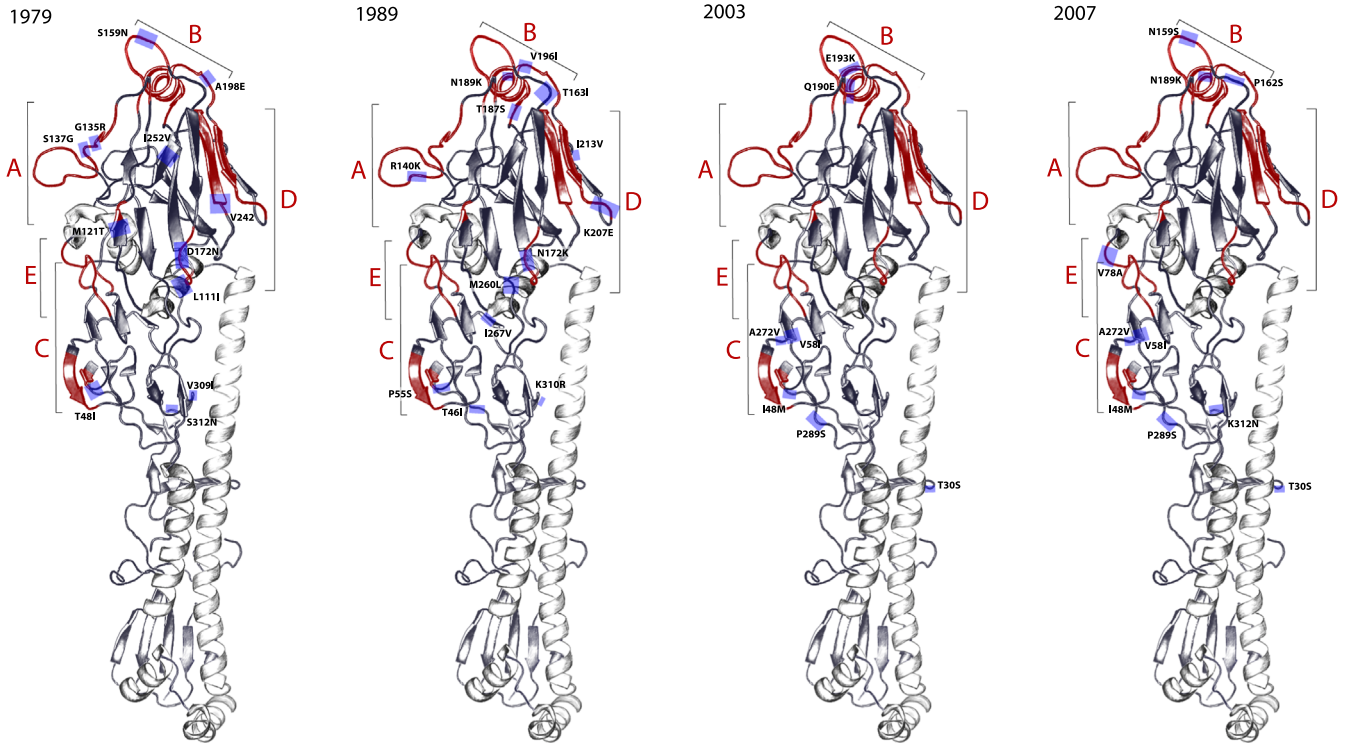

Fig. 6. EIV epidemics: HA monomeric structures. The location of HA1 amino acid substitutions between vaccine or previous epidemic strains and outbreak isolates are highlighted with blue boxes for large- scale outbreaks in 1979 (UK), 1989 (UK), 2003 (UK) and 2007 (Japan). The positions of antigenic sites identified for human H3N2 viruses are indicated in red, from Wiley et al. (1981), Wiley and Skehel (1987) and Wilson and Cox (1990). Antigenic site A: 122, 128, 132–146; site B: 155–160, 186–199; site C: 50, 53–54, 91–92, 275–278; site D: 172–174, 201–207, 217–220, 242–248; site E: 62–63, 78–83. Diagrams were redrawn using Adobe Photoshop CS6.6 from structures viewed in MacPyMOL.

also increased the titre against the American antiserum to Newmarket/1/93. This mutation also caused a subtle increase (three fold) in recognition by antiserum to the vaccine strain Fontainebleau/79 but not Newmarket/79. This mutation occurs within the 220 loop region of the receptor-binding site (Fig. 4). Taken together, these data suggest that, like residue 159, positions 189 and 227 may also be antigenically important for EIV.

Antigenicity of Sussex/89 point mutants

In this converse series of experiments, point mutants generated in the Sussex/89 HA background were analysed using the same panel of ferret antisera as that used for the Newmarket/79 mutants. The aim was to identify single amino acid substitutions that would either increase recognition by antisera raised against the vaccine strains Newmarket/79 and Fontainebleau/79 or decrease recognition by the Eurasian antisera (Sussex/89, Yvelines/89, Newmarket/2/93). All HI assays were carried out against wild type Sussex/89 HA rescued in the Kentucky/02 reverse genetics background as a control. The WT Sussex/89 7:1 reassortant gave slightly lower titres overall than the parental virus, but a similar pattern of reactivity was seen against the Eurasian antisera (Tables 1 and 2), indicating that it was antigenically similar and a suitable backbone to use for mutagenesis. Three of the four Eurasian sera gave titres of more than 128, however Hong

Kong/92 gave a relatively low GMT titre of 26. This was similar to the pattern seen for the parental viruses, where the serum for Hong Kong/92 gave a lower titre against Sussex/89 than most of the Eurasian sera (Table 1). The Sussex/89 WT virus was not recognised by serum to the Newmarket/79 vaccine strain (GMT value <8) but gave a measurable titre against Fontainebleau/79, reflecting the antigenic difference between the two vaccine strains.

As for the previous set of point mutations in the Newmarket/79 background, most of the mutations had little effect on recognition of Sussex/89. All the Sussex/89 mutant viruses had low titres to the Newmarket/79 ferret antisera, ranging from <8 up to a maximum of 16. Subtle increases in titre (around three fold) to this serum were observed with the N159D/I163T double mutant and with E207K, however these results indicated that no single mutation was sufficient for the mutants to resemble the WT Newmarket/79 strain antigenically. Interestingly, neither the single or double 159 Sussex/89 mutants were recognised by Fontainebleau/79 serum (Tables 2 and S2), consistent with the antigenic importance of 159N for this strain. Unlike the Newmarket/79 serum, Fontainebleau/79 serum recognised most of the other Sussex/89 point mutants to a fairly consistent titre of around 64 (Table 2). This suggested further that the amino acid differences between the two vaccine strains (Fig. 1) were antigenically significant.

Striking results were obtained with the mutation K189N in the Sussex/89 background, which specifically blocked recognition by all four of the ferret antisera raised against the Eurasian viruses, without changing the ability of the antisera raised against the American lineages viruses to bind (Table 2). Two further Sussex/89 mutants showed obvious differences in recognition by Eurasian sera compared to WT, with changes at 159 and 227 both reducing HI titres by more than 4-fold against one or more serum. This correlated to some extent with the increase in recognition seen in mutants with the reciprocal changes D159N and P227S in the Newmarket/79 background described above, however decreased titres were seen against a broader selection of sera. For instance the S227P mutation within the Sussex/89 background led to decreased recognition by all the sera in the panel (Table 2). N159D had a more specific effect, but also affected recognition by sera raised against all 4 sub-lineages tested. Both of these mutants demonstrated reduced recognition by American antisera raised to Kentucky and FC2 viruses, although the titres for WT Sussex/89 were relatively low to start with.

To summarise, three mutations at positions 159, 189 and 227 all resulted in 4-fold or greater differences in HI titre, the positions of these changes with respect to the receptor binding site is shown in Fig. 4.

Analysis of HA1 from large-scale influenza outbreaks

To extend the analysis above, we compared the HA1 amino acid sequence data of isolates from other known instances of vaccine breakdown or countrywide outbreaks of equine influenza against their respective vaccine strains or previous epidemic strains, arguing that herd immunity to the previous circulating virus was also relevant for the analysis of antigenic drift. The examples used were: UK 1979 (previous circulating viruses and vaccine strains dated from the early 1960s), UK 1989 described here, UK 2003 (vaccine strains Newmarket/1/93, Newmarket/2/93, previous outbreak Sussex/1989), and Japan 2007 (vaccine strains Avesta/1993, La Plata/1993). Each outbreak is summarised in Fig. 5, which shows the sub-lineage responsible, the relevant vaccine strains and the amino acid differences between them, together with their location with respect to known antigenic sites in human H3. To identify amino acid substitutions relevant for the UK outbreak in 1979, 15 complete equine H3N8 HA1 amino acid sequences available from the Influenza Virus Resource at NCBI (NCBI-IVR, Bao et al., 2008) from 1963 to 1980 were aligned (Fig. S2). Eight or more substitutions were observed between pandemic strains from the 1960s and those from 1976 to 1979. A similar alignment was generated for the 1989 outbreak (Supplementary data, Fig. S3); alignments for the 2003 and 2007 outbreaks have been reported previously (Newton et al., 2006; Ito et al., 2008), alignments of HA1 sequences currently available from the NCBI-IVR are provided in Figs. S3 and S4. In most instances, amino acid substitutions between outbreak and earlier vaccine or epidemic strains were clear, for others the amino acid residue at a given position was more variable between different isolates (Figs. S2 and S3). Amino acid substitutions that may have been important for antigenic drift in each outbreak are summarised in Fig. 5. To determine which changes occurred within antigenic sites, the positions of the relevant substitutions were mapped onto the HA1 monomer, using the PDB structure of A/duck/Ukraine/63 (Fig. 6). For simplicity, antigenic sites were collated from those described by Wiley et al. (1981), Wiley and Skehel (1987) and Underwood et al. (1987) and are indicated in Fig. 6. All four outbreaks involved amino acid changes in multiple locations and within antigenic site B and at least one other. The two earlier outbreaks (1979 and 1989) had changes in the loop regions associated with antigenic sites A and D (Fig. 6), the two later outbreaks (2003 and 2007) had changes in sites C or E. Several changes were located in loop regions close to antibody binding sites for human H3 viruses, but not actually included in these sites, such as T46I, A272V and P289S near site C and L111I and M260L near site D. Interestingly, 3 of the 4 outbreaks also involved changes in an additional loop close to antigenic site C: V309I, K310R or K312N. Amino acid substitutions at positions 48, 172, 189 and 312 were associated with more than one outbreak. Overall, comparison of four major outbreaks shows that at least 8 amino acid differences were observed, affecting 2 or more of the antigenic sites defined for human H3, and several substitutions were identified that occurred outside but close to previously defined antibody binding sites. These figures were consistent with results of the detailed reverse genetics investigation carried out here on the UK 1989 outbreak, which suggested that at least three amino acid substitutions contributed to the overall antigenic difference between outbreak and vaccine strains. The substitutions at positions 159 and 189 were located within antigenic site B and that at position 227 occurs close to antigenic site D. All three were close to or within the receptor-binding site of HA (Fig. 4).

Discussion

Equine influenza viruses, like other influenza A viruses, undergo antigenic drift as a result of amino acid substitutions in HA (Daniels et al., 1985). Analyses of human H3N2 epidemics led to the conclusion that two or more amino acid substitutions in two or more antigenic sites were sufficient for escape from pre-existing immunity (Wilson and Cox, 1990). In comparison, reports suggest that EIV does not undergo antigenic drift at the same rate as human influenza (Murcia et al., 2011; Lewis et al., 2011). It is also not clear how many substitutions, where, or of what type, are required before equine influenza vaccine breakdown is likely to occur in the field. Previous studies of the 1989 and 2007 outbreaks showed that vaccines failed to protect from EIV infection when there were between 10 and 16 amino acid differences between them (Ito et al., 2008, Binns et al., 1993). In terms of antigenic analyses, antigenic cartography was applied to EIV and suggested that one or two substitutions in key sites may be sufficient to cause an antigenic 'cluster jump' (Lewis et al., 2011). Similar results were reported for human influenza viruses, suggesting that a very limited repertoire of amino acid substitutions was responsible for antigenic drift (Smith et al., 2004; Koel et al., 2013). Complications in antigenic

analyses can arise as a result of one-way cross-reactivity in HI assays, such as that shown here between sera raised against the 1979 vaccine strains against the emergent outbreak strains from 1989 (Table 1). Similar patterns of one-way cross reactivity have been shown between vaccine strains belonging to the Kentucky sublineage and outbreak viruses from the Florida sublineage (Bryant et al., 2009, 2011). This phenomenon of sera against vaccine strains continuing to recognise field strains, even those known to have caused large scale vaccine breakdown, highlights the importance of including sera raised against current strains for HI analysis.

Here we have taken an important example of EIV vaccine breakdown in the field, the 1989 incursion of the Eurasian sub-lineage in the UK, and applied reverse genetics and site directed mutagenesis to facilitate an in depth study of the importance of each amino acid substitution between vaccine and outbreak strains. The original analyses identified 16 amino acid differences (Binns et al., 1993); by increasing the number of sequences compared, we reduced this number to 13 conserved differences between strains with a further two substitutions between UK outbreak and vaccine strains. We found that single amino acid substitutions at positions 159, 189 and 227 all affected the recognition of mutant viruses by ferret antisera. The most striking differences were observed with substitution K189N in the Sussex/89 background and the reciprocal change N189K in the Newmarket/79 virus. This amino acid occurs within antigenic site B, which underwent substitution in all four major outbreaks of EIV described here. It is also within the 190 helix of HA, comprising part of the receptor binding site for sialic acid for H3 viruses (Fig. 4). Site B has previously been suggested as the major antigenic site for equine influenza viruses (Daly et al., 1996). Our previous antigenic cartography data indicated that K189 was likely to be important for distinguishing the Eurasian sub-lineage from viruses with 189-D, Q or E (Lewis et al., 2011), switching of the amino acid between uncharged, acidic and basic at position 189 thus appears to be an important factor in altering antigenic properties of EIV. Change in the residue at position 189 was also shown to be important for antigenic drift in human and swine H3N2 viruses (Koel et al., 2013, Ye et al., 2013, Lewis et al., 2014) and was associated with human vaccine breakdown in Iran during the 2005–2006 influenza season (Moattari et al., 2010).

Two substitutions in the Newmarket/79 HA, D159N and P227S, both increased recognition by antisera raised against Fontainebleau/79 and the reciprocal changes in a Sussex/89 background reduced recognition by Eurasian sera. Residue 227 is within the 220 loop of the receptor-binding site of HA (Fig. 4), the substitution P227S may therefore have had an indirect effect on the HI assay by altering receptor-binding activity. However, the effect on HI titres shown here was specific to a subset of the antisera used, suggesting that this did not result in a non-specific effect on avidity. In support of substitution at this position having antigenic potential, residue 227 was assigned to antigenic site D of human H3 by Stray and Pittman (2012), it has previously been implicated in recognition of human H3N2 by neutralising monoclonal antibodies (Vanlandschoot et al., 1995) and it underwent substitution in recent human H3N2 isolates (Bragstad et al., 2008). Alteration of the residue at position 159, located within antigenic site B, was one of three differences between the vaccine strains Fontainebleau/79 and Newmarket/79 and had a profound effect on antibody recognition. 159 is located within the 150 loop of HA, a region that is important for recognition by a broadly neutralising monoclonal antibody against human H3 (Lee et al., 2012). 159D found in Newmarket/79 was atypical of other strains isolated during this period (Fig. S2), which all had 159N. Substitution at this position may have been a component of antigenic drift between the 1979 viruses and the original H3N8 viruses circulating in the early 1960s (Fig. 3, Fig. S2, Daniels et al., 1985). Although we cannot pinpoint this residue as being significant for the UK outbreak in 1989, the substitution N159S is one of those distinguishing the current FC1 and FC2 sub-lineages (Bryant et al., 2009; Lewis et al., 2011) and also featured in the EIV outbreak in Japan in 2007 (Fig. 6, Ito et al., 2008). Early representatives of the FC1 and FC2 sub-lineages differed by only two amino acids in HA, at positions V78A (site E) and N159S, yet showed a 4–16 fold difference in HI titre against ferret antisera (Bryant et al., 2009). This was despite relatively conservative substitutions, i.e. no changes in charge or polarity, in agreement with the suggestion that the major factor for human H3 viruses is frequency of change in a particular residue, rather than necessarily the type of change (Stray and Pittman, 2012). As substitution at 159 may be important for evolution of more than one sub-lineage of EIV, this may play as important a role as 189 in antigenic drift for EIV.

The antigenic characterisation of single point mutants described above suggests that only a few amino acid changes in antigenic sites may be needed to cause a detectable antigenic change in equine HA, as found by Jin et al. (2005) and Koel et al. (2013) for human H3 viruses. However, epidemiological evidence from field outbreaks of EIV (Figs. 5 and 6) shows that sizeable outbreaks of equine influenza have only occurred in the face of multiple amino acid changes. This is in general agreement with data reported for human H3N2 viruses, where epidemics and vaccine breakdown occurred in the face of multiple changes in HA (Wiley et al., 1981; Wilson and Cox, 1990; Jin et al., 2005). Interestingly, recent reports of antigenic drift amongst human H3N2 viruses away from the current WHO vaccine strain recommendations show that the drift variants have six or seven changes affecting antigenic sites A, B and D, including substitution at position 159 (WHO, September 2014 recommendations).

We found that at least 8 amino acid substitutions were associated with major outbreaks, with changes in antigenic site B and at least one other, thus the pattern did not necessarily comply with the 'four or more changes located in two or more antigenic sites' guideline that arose from analyses of human epidemics (Wilson and Cox, 1990). Earlier studies concluded that at least one change in each of the antigenic sites appeared to be required (Wiley et al., 1981, Wiley and Skehel, 1987). For EIV epidemics, in some cases, only one change was observed in a given site and, in several instances, amino acid residues close to the antigenic sites showed substitutions between vaccine and outbreak strains but had not previously been assigned to those sites. In particular, multiple changes were located within the loop regions associated with antigenic sites C and D (residues 48, 272, 273, 276). Multiple changes also occurred in a loop region near the C-terminus of HA1: 309, 310, 312.

More information is required on the contribution of other influenza virus proteins, including NA, to protective immunity induced by natural infection and vaccination before we can predict the influence of antigenic drift with accuracy. The data presented here identify some key amino acid changes that have a pronounced effect; however, HI analysis can only detect those changes that directly or indirectly affect binding of antibodies that block the interaction between HA1 and sialic acid receptors. The three changes we have highlighted all map close to the receptor binding site, as do those mapped by Koel et al. (2013) and Lewis et al. (2014). Two of the three changes (159, 189) involved different substitutions but at the same locations as those highlighted for human H3N2 viruses (Koel et al., 2013) and swine H3N2 viruses (Lewis et al., 2014). The assays employed are unlikely to detect other relevant changes that could alter binding by other neutralising antibodies. For instance, the change P273L in antigenic site C was associated with the Eurasian viruses responsible for the 1989 outbreaks and did not have an effect on the HI titres of the antisera tested here, however this substitution was also detected in a monoclonal antibody escape mutant of human H3N2 (Smith et al., 1991). This type of analysis also does not consider the importance of T-cell epitopes, which are known to exist in HA (Babon et al., 2009; Duvvuri et al., 2010, Jameson et al., 1998). Interestingly the region near the C-terminus of HA1, including

residues 309–312 highlighted above, contained an immunodominant epitope recognised by human T helper cells isolated from people exposed to H3 viruses (Lamb and Green, 1983). Two examples of major EIV epidemics described here involved a vaccine strain mismatch in this region. Detailed surveillance and careful investigation of large-scale epidemics in the future, whether due to breakdown of natural or vaccine-induced immunity, will aid the refinement of vaccine strain selection for EIV.

Methods

Viruses

Low passage equine influenza virus isolates from the archive at the Animal Health Trust 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 haemagglutination assay (HA) using 1% chicken erythrocytes in PBS according to standard methods (OIE, 2012). Viruses were serially passaged until stable titres were obtained, up to a maximum of three passages.

Plasmids

The plasmid pPolI-SapRZ, containing a human polI promoter and delta ribozyme with SapI cloning sites, was kindly provided by Professor Ervin Fodor, University of Oxford. The EIV reverse genetics system for A/equine/Kentucky/02 (Quinlivan et al., 2005) was kindly provided by Professor Peter Palese, Mount Sinai School of Medicine, New York. Expression plasmids encoding influenza proteins PB1, PB2, PA and NP from A/equine/Richmond/07 under the control of the CMV IE promoter were constructed by ligating PCR products amplified by RT-PCR from genomic viral RNA, using standard techniques. Primer sequences are available on request.

Sequencing

Viral RNA was isolated from 140 μl allantoic fluid using the QIAamp Viral RNA mini kit (Qiagen) according to the manufacturer's instructions and eluted in 60 μl. Sequencing of segment 4, encoding HA, was completed using overlapping PCR fragments. Briefly, cDNA was reverse transcribed using Superscript II enzyme (Life Technologies) with influenza A Uni-12 primer (Hoffmann et al., 2001) according to manufacturer's instructions. The HA gene was amplified in the form of four overlapping segments of approximately 500 nucleotides by PCR using native PFU DNA polymerase (Stratagene) and equine H3 HA-specific primers combined with M13 forward or reverse sequences as described by Rash et al. (2014). Samples were sequenced using ABI BigDye® Terminator v3.1 (Applied Biosystems) on an ABI PRISM® 3100 Genetic Analyser (Applied Biosystems). Nucleotide sequences were visualised and edited using Seqman II version 5.03 (DNAstar Inc).

Reverse genetics

Viral genome segments for the equine influenza strains A/equine/Sussex/89 and A/equine/Newmarket/79 were reverse-transcribed from purified viral RNA using Superscript II (Invitrogen) and uni-12 primer, 5′-AGCGAAAGCAGG-3′. Segment 4, encoding HA, was then amplified by PCR with equine influenza HA-specific primers: ERG HA cRNA 5′-CGA T**GC TCT TC**T GCC **AGC AAA AGC AGG GGA TAT TTC TGT C-3**′ and ERG HA vRNA 5′-CGA T**GC TCT TC**T ATT **AGT AGA AAC AAG GGT GTT TTT AAC-**3′. PCR products were purified, digested with *Sap*I and ligated into pPolISapRZ using standard techniques to generate the plasmid pPolI-HA for both Sussex/89 and Newmarket/79. Plasmid constructs were sequenced and the data analysed as described above. Recombinant viruses were rescued following transfection of 293T cells with 12 plasmids: pPolI-HA, 7 plasmids encoding the remaining EIV genome segments from the Kentucky/02 reverse genetics system (Quinlivan et al., 2005) and 4 expression plasmids containing the coding sequences for PB1, PB2, PA and NP from EIV strain A/equine/Richmond/07 ligated into pcDNA3. Plasmids were purified from bacterial cultures using a Qiagen Maxi prep kit and diluted to 0.5 μg/μl in water then used to transfect 293 T cells in suspension. Briefly, 10 μl Lipofectin and 100 μl Optimem were mixed per plasmid mixture, according to manufacturer's instructions, using 1 μg each plasmid construct per transfection. Plasmids were gently added to 1 ml 293T cells diluted to 1×10^6 per ml in serum-free Dulbecco's Modified Eagles Medium (DMEM) (Lonza) then cells incubated in 6-well plates at 37 °C, 5% CO_2 overnight. Transfection supernatant was replaced with 2 ml of warmed overlay media, consisting of serum-free DMEM with 1% Penicillin/Streptomycin. 1% L/G, 0.2% Trypsin (Worthington) and 0.14% BSA. Plates were incubated for a further 2 days, culture supernatants were clarifed by low speed centrifugation then 100 μl inoculated into fertile hens' eggs, as described above for growth of EIV strains.

Haemagglutination inhibition assays

Antigenic characterisation was conducted using haemagglutination inhibition (HI) assays with ferret and equine antisera as previously described (Bryant et al., 2009, Woodward et al., 2014). For parental viruses, geometric mean titres (GMT) were calculated from three HI tests for each combination. For recombinant viruses generated by reverse genetics and mutagenesis, GMTs were calculated from a minimum of three and maximum of eight HI assays. Viruses were tested against post-infection antisera raised against representative strains from the different sub-lineages of EIV: Pre-divergence, American/Kentucky, Eurasian, Florida clade 1 and Florida clade 2.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.02.027.

References

Babon, J.A., Cruz, J., Orphin, L., Pazoles, P., Co, M.D., Ennis, F.A., Terajima, M., 2009. Genome-wide screening of human T-cell epitopes in influenza A virus reveals a broad spectrum of CD4(+) T-cell responses to internal proteins, hemagglutinins, and neuraminidases. Hum. Immunol. 70, 711–721.

Baigent, S.J., McCauley, J.W., 2001. Glycosylation of haemagglutinin and stalk-length of neuraminidase combine to regulate the growth of avian influenza viruses in tissue culture. Virus Res. 79, 177–185.

Bao, Y., Bolotov, P., Dernovoy, D., Kiryutin, B., Zaslavsky, L., Tatusova, T., Ostell, J., Lipman, J. & D., 2008. The Influenza Virus Resource at the national Center for Biotechnology Information. J. Virol. 82, 596–601.

Barbic, L., Madic, J., Turk, N., Daly, J., 2009. Vaccine failure caused an outbreak of equine influenza in Croatia. Vet. Microbiol. 133, 164–171.

Binns, M.M., Daly, J.M., Chirnside, E.D., Mumford, J.A., Wood, J.M., Richards, C.M., Daniels, R.S., 1993. Genetic and antigenic analysis of an equine influenza H3 isolate from the 1989 epidemic. Arch. Virol. 130, 33–43.

Blackburne, B.P., Hay, A.J., Goldstein, R.A., 2008. Changing selective pressure during antigenic changes in human influenza H3. PLoS Pathog. 4 (5)e1000058.
Bragstad, K., Nielsen, L.P., Fomsgaard, A., 2008. The evolution of human influenza A viruses from 1999 to 2006: A complete genome study. Virol. J. 5, 40.
Bryant, N.A., Rash, A.S., Russell, C.A., Ross, J., Cooke, A., Bowman, S., MacRae, S., Lewis, N.S., Paillot, R., et al., 2009. Antigenic and genetic variations in European and North American equine influenza virus strains (H3N8) isolated from 2006 to 2007. Vet. Microbiol. 138, 41–52.
Bryant, N.A., Rash, A.S., Woodward, A.L., Medcalf, E., Helwegen, M., Wohlfender, F., Cruz, F, Herrmann, C., Borchers, K., Tiwari, A., Chambers, T.M., Newton, J.R., Mumford, J.A., Elton, D.M., 2011. Isolation and characterisation of equine influenza viruses (H3N8) from Europe and North America from 2008 to 2009. Vet. Microbiol. 147, 19–27.
Burrows, R., Denyer, M., Goodridge, D., Hamilton, F., 1981. Field and laboratory studies of equine influenza viruses isolated in 1979. Vet. Rec. 109, 353–356.
Burrows, R., Denyer, M., 1982. Antigenic properties of some equine influenza viruses. Arch. Virol. 73, 15–24.
Daly, J.M., Lai, A.C.K., Binns, M.M., Chambers, T.M., Barrandeguy, M., Mumford, J.A., 1996. Antigenic and genetic evolution of equine H3N8 influenza A viruses. J. Gen. Virol. 77, 661–671.
Daniels, R.S., Skehel, J.J., Wiley, D.C., 1985. Amino acid sequences of haemagglutinins of influenza viruses of the H3 subtype isolated from horses. J. Gen. Virol. 66, 457–464.
de Jong, J.C., Smith, D.J., Lapedes, A.S., Donatelli, I., Campitelli, L., Barigazzi, G., Van Reeth, K., Jones, T.C., Rimmelzwaan, G.F., et al., 2007. Antigenic and genetic evolution of swine influenza A (H3N2) viruses in Europe. J. Virol. 81, 4315–4322.
Duvvuri, V.R., Moghadas, S.M., Guo, H., Duvvuri, B., Heffernan, J.M., Fisman, D.N., Wu, G.E., Wu, J., 2010. Highly conserved cross-reactive CD4+ T-cell HA-epitopes of seasonal and the 2009 pandemic influenza viruses. Influenza Other Respir. Viruses 4, 249–258.
Ha, Y., Stevens, D.J., Skehel, J.J., Wiley, D.C., 2003. X-ray structure of the hemagglutinin of a potential H3 avian progenitor of the 1968 Hong Kong pandemic influenza virus. Virology 309, 209–218.
Hinshaw, V.S., Naeve, C.W., Webster, R.G., Douglas, A., Skehel, J.J., Bryans, J., 1983. Analysis of antigenic variation in equine 2 influenza A viruses. Bull. World Health Organ. 61, 153–158.
Hoffmann, E., Stech, J., Guan, Y., Webster, R.G., Perez, D.R., 2001. Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. 146, 2275–2289.
Ito, M., Nagai, M., Hayakawa, Y., Komae, H., Murakami, N., Yotsuya, S., Asakura, S., Sakoda, Y., Kida, H., 2008. Genetic analyses of an H3N8 Influenza virus isolate, causative strain of the outbreak of equine influenza at the Kanazawa Racecourse in Japan in 2007. J. Vet. Med. Sci. 70, 899–906.
Jameson, J., Cruz, J., Ennis, F.A., 1998. Human cytotoxic T-lymphocyte repertoire to influenza A viruses. J. Virol. 72, 8682–8689.
Jin, H., Zhou, H., Liu, H., Chan, W., Adhikary, L., Mahmood, K., Lee, M.-S., Kemble, G., 2005. Two residues in the hemagglutinin of A/Fujian/411/02-like influenza viruses are responsible for antigenic drift from A/Panama/2007/99. Virology 336, 113–119.
Kaverin, N.V., Gambaryan, A.S., Bovin, N.V., Rudneva, I.A., Shilov, A.A., Khodova, O.M., Varich, N.L., Sinitsin, B.V., Makarova, N.V., Kropotkina, E.A., 1998. Postreassortment changes in influenza A virus hemagglutinin restoring HA-NA functional match. Virology 244, 315–321.
Kobayashi, Y., Suzuki, Y., 2012. Compensatory evolution of net-charge in influenza A virus hemagglutinin. PLoS One 7 (7), e40422.
Koel, B.F., Burke, D.F., Bestebroer, T.M., van der Vliet, S., Zondag, G.C., Vervaet, G., Skepner, E., Lewis, N.S., Spronken, M.I., et al., 2013. Substitutions near the receptor binding site determine major antigenic change during influenza virus evolution. Science 342, 976–979.
Lai, A.C., Lin, Y.P., Powell, D.G., Shortridge, K.F., Webster, R.G., Daly, J., Chambers, T.M., 1994. Genetic and antigenic analysis of the influenza virus responsible for the 1992 Hong Kong equine influenza epizootic. Virology 204, 673–679.
Lai, A.C., Chambers, T.M., Holland Jr., R.E., Morley, P.S., Haines, D.M., Townsend, H.G., Barrandeguy, M., 2001. Diverged evolution of recent equine-2 influenza (H3N8) viruses in the Western Hemisphere. Arch. Virol. 146, 1063–1074.
Lamb, J.R., Green, N., 1983. Analysis of the antigen specificity of influenza haemagglutinin-immune human T lymphocyte clones: identification of an immunodominant region for T cells. Immunology 50, 659–666.
Lee, P.S., Yoshida, R., Ekiert, D., Sakai, N., Suzuki, Y., Takada, A, 2012. Heterosubtypic antibody recognition of the influenza virus hemagglutinin receptor binding site enhanced by avidity. Proc. Natl. Acad. Sci. USA 109, 17040–17045.
Lewis, N.S., Anderson, T.K., Kitikoon, P., Skepner, E., Burke, D.F., Vincent, A.L., 2014. Substitutions near the hemagglutinin receptor-binding site determine the antigenic evolution of influenza A H3N2 viruses in U.S. swine. J. Virol. 88, 4752–4763.
Lewis, N.S., Daly, J.M., Russell, C.A., Horton, D.L., Skepner, E., Bryant, N.A., Burke, D.F., Rash, A.S., Wood, J.L., et al., 2011. Antigenic and genetic evolution of equine influenza A (H3N8) virus from 1968 to 2007. J. Virol. 85, 12742–12749.
Lin, Y.P., Xiong, X., Wharton, S.A., Martin, S.R., Coombs, P.J., Vachieri, S.G., Christodoulou, E., Walker, P.A., Liu, J., Skehel, J.J., Gamblin, S.J., Hay, A.J., Daniels, R.S., McCauley, J.W., 2012. Evolution of the receptor binding properties of the influenza A(H3N2) hemagglutinin. Proc. Natl. Acad. Sci. USA 109, 21474–21479.
Livesay, G.J., O'Neill, T., Hannant, D., Yadav, M.P., Mumford, J.A., 1993. The outbreak of equine influenza (H3N8) in the United Kingdom in 1989: diagnostic use of an antigen capture ELISA. Vet. Rec. 133, 515–519.
Mitnaul, L.J., Matrosovich, M.N., Castrucci, M.R., Tuzikov, A.B., Bovin, N.V., Kobasa, D., Kawaoka, Y., 2000. Balanced hemagglutinin and neuraminidase activities are critical for efficient replication of influenza A virus. J. Virol. 74, 6015–6020.
Moattari, A., Ashrafi, H., Kadivar, M.R., Kheiri, M.T., Shahidi, M., Arabpour, M., Ghanbari, A., 2010. Antigenic variations of human influenza virus in Shiraz, Iran. Indian J. Med. Microbiol. 28, 114–119.
Murcia, P.R., Wood, J.L., Holmes, E.C., 2011. Genome-scale evolution and phylodynamics of equine H3N8 influenza A virus. J. Virol. 85, 5312–5322.
Nelson, M.I., Holmes, E.C., 2007. The evolution of epidemic influenza. Nat. Rev. Genet. 8, 196–205.
Newton, J.R., Daly, J.M., Spencer, L., Mumford, J.A., 2006. Description of the outbreak of equine influenza (H3N8) in the United kingdom in 2003, during which recently vaccinated horses in Newmarket developed respiratory disease. Vet. Rec. 158, 185–192.
OIE, 2012. Equine influenzaManual of Diagnostic Tests and Vaccines for Terrestrial Animals (Mammals, Birds and Bees)7th ed. OIE Biological Standards Commission, World Organisation for Animal Health (OIE), Paris, pp. 865–878, (Chapter 2.5.7).
Oxburgh, L., Berg, M., Klingeborn, B., Emmoth, E., Linné, T., 1993. Equine influenza virus from the 1991 Swedish epizootic shows major genetic and antigenic divergence from the prototype virus. Virus Res. 28, 263–272.
Powell, D.G., Watkins, K.L., Li, P.H., Shortridge, K.F., 1995. Outbreak of equine influenza among horses in Hong Kong during 1992. Vet. Rec. 136, 531–536.
Quinlivan, M., Zamarin, D., Garcia-Sastre, A., Cullinane, A., Chambers, T., Palese, P., 2005. Attenuation of equine influenza viruses through truncations of the NS1 protein. J. Virol. 79, 8431–8439.
Rash, A., Woodward, A., Bryant, N., McCauley, J., Elton, D., 2014. An efficient genome sequencing method for equine influenza [H3N8] virus reveals a new polymorphism in the PA-X protein. Virol. J. 11, 159.
Rose, M.A., 1966. Respiratory virus infections in man and animals. Equine influenza viruses isolated at Cambridge in 1963 and 1965. Proc. R. Soc. Med. 59, 51–54.
Smith, C.A., Barnett, B.C., Thomas, B., Temoltzin-Palacios, F., 1991. Structural assignment of novel and immunodominant antigenic sites I the neutralizing antibody response of CBA/Ca mice to influenza haemagglutinin. J. Exp. Med. 173, 953–959.
Smith, D.J., Lapedes, A.S., de Jong, J.C., Bestebroer, T.M., Rimmelzwaan, G.F., Osterhaus, A.D.M.E., Fouchier, R.A.M., 2004. Mapping the antigenic and genetic evolution of influenza virus. Science 305, 371–376.
Stray, S.J., Pittman, L.B., 2012. Subtype- and antigenic site-specific differences in biophysical influences on evolution of influenza virus hemagglutinin. Virol. J. 9, 91.
Underwood, P.A., Skehel, J.J., Wiley, D.C., 1987. Receptor-binding characteristics of monoclonal antibody-selected antigenic variants of influenza virus. J. Virol. 61, 206–208.
Vanlandschoot, P., Beirnaert, E., Dewilde, S., Saelens, X., Bestebroer, T., De Jong, J., Jou, W.M., Fiers, W., 1995. A fairly conserved epitope on the hemagglutinin of influenza A (H3N2) virus with variable accessibility to neutralizing antibody. Virology 212, 526–534.
World Health Organisation , 2002. In: The World Health Report 2002: Reducing Risks, Promoting Healthy Life. Edited by World Health Organisation, Geneva, Switzerland.
World Health Organisation, (September 2014 recommendations): ⟨http://www.who.int/influenza/vaccines/virus/recommendations/⟩.
Wilson, I.A., Cox, N.J., 1990. Structural basis of immune recognition of influenza virus hemagglutinin. Annu. Rev. Immunol. 8, 737–771.
Wiley, D.C., Wilson, I.A., Skehel, J.J., 1981. Structural identification of the antibody binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. Nature 289, 373–378.
Wiley, D.C., Skehel, J.J., 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annu. Rev. Biochem. 56, 365–394.
Woodward, A.L., Rash, A.S., Blinman, D., Bowman, S., Chambers, T.M., Daly, J.M., Damiani, A., Joseph, S., Lewis, N., et al., 2014. Development of a surveillance scheme for equine influenza in the UK and characterisation of viruses isolated in Europe, Dubai and the USA from 2010 to 2012. Vet. Microbiol. 169, 113–127.
Ye, J., Xu, Y., Harris, J., Sun, H., Bowman, A.S., Cunningham, F., Cardona, C., Yoon, K.J., Slemons, R.D., Wan, X.F., 2013. Mutation from arginine to lysine at the position 189 of hemagglutinin contributes to the antigenic drift in H3N2 swine influenza viruses. Virology 446, 225–229.