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Short communication

Vaccine failure caused an outbreak of equine influenza in Croatia

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Abstract

In April 2004 an outbreak of equine influenza occurred at the Zagreb hippodrome, Croatia. Clinical respiratory disease of the same intensity was recorded in vaccinated and non-vaccinated horses. The equine influenza vaccine used in Croatia at the time of the outbreak contained the strains A/equine/Miami/63 (H3N8), A/equine/Fontainebleau/79 (H3N8) and A/equine/Prague/56 (H7N7). At the same time, the usual strains in vaccines used in Europe were, in accordance with the recommendation of the World Organisation for Animal Health (OIE) Expert Surveillance Panel on equine influenza, A/equine/Newmarket/1/93 (H3N8) and A/equine/Newmarket/2/93 (H3N8). At the same time, some current vaccines in the USA contained A/equine/Kentucky/97 (H3N8). Genetic characterization of the HA1 portion of the haemagglutinin (HA) gene of virus isolated from the outbreak indicated that the isolate (A/equine/Zagreb/04) was an H3N8 strain closely related to recent representative viruses of the American lineage Florida sub-lineage. In comparison with both H3N8 vaccine strains used in horses at the Zagreb hippodrome, A/equine/Zagreb/04 displayed amino acids changes localised to 4 of the 5 described antigenic sites (A–D) of subunit protein HA1. Comparison of the amino acid sequence of the HA1 subunit protein of the outbreak strain with that of A/equine/Newmarket/1/93 displayed three amino acids changes localised in antigenic sites B and C, while antigenic sites A, D and E were unchanged. The Zagreb 2004 outbreak strain had the same amino acids at antigenic sites of the HA1 subunit protein as the strain A/equine/Kentucky/97. Amino acid changes in antigenic sites between HA1 subunit of the outbreak strain and the strains used in the vaccines likely accounted for the vaccine failure and the same clinical signs in vaccinated and unvaccinated horses. Use of a recent strain in vaccines should limit future outbreaks.
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Keywords: Horse; Equine influenza; Outbreak; Vaccination; Protection; Antigenic drift

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1. Introduction

Equine influenza is a major respiratory disease in horses worldwide, caused by a type A influenza virus, family *Orthomyxoviridae*. Two different subtypes of equine influenza virus, H7N7 and H3N8, have been associated with disease in the horse. Studies of the evolution of H3N8 subtype equine influenza viruses suggested evolution in a single lineage (Kawaoka et al., 1989) until the late 1980s when American and European lineage of H3N8 viruses emerged (Daly et al., 1996). Strains within the American lineage further diverged into three distinct sub-lineages, namely South American, Florida and Kentucky (Lai et al., 2004). These lineages and sub-lineages are antigenically and genetically distinguishable from one other as a result of antigenic drift in the gene coding for the haemagglutinin (HA), the major surface protein of influenza A viruses (Daly et al., 1996; Lai et al., 2004). Mutation of the gene coding for HA may occur in one or more of five major antigenic sites (Wiley and Skehel, 1987) on the HA1 subunit protein, which can lead to the virus not being recognisable by pre-existing antibodies generated by infection or vaccination with an earlier strain (Daly et al., 2003). Studies that analysed the relationship between vaccine-induced antibody level measured with the haemagglutination inhibition (HI) test and protection against infection were unclear and HI titres ranging from 8 to 128 were quoted as being protective (Daly et al., 2004). In contrast, serum levels of vaccine-induced SRH antibody correlated closely with protective immunity against challenge (Hannant et al., 1988) and in different experiments were between 120 and 154 mm^2 for virological protection (Mumford, 1992) and $\geq$80–85 mm^2 for clinical protection (Mumford et al., 1988, 1990; Heldens et al., 2004).

After realising that H3N8 strains had diverged into two distinct evolutionary lineages (Daly et al., 1996), the WHO/OIE experts formed an Expert Surveillance Panel and it was recommended that vaccines should contain one H3N8 strain representative of the American lineage and a representative of the European lineage (OIE, 1996). Despite the recommendations vaccine containing strains A/equine/Miami/63 (H3N8), A/equine/Fontainebleau/79 (H3N8) and A/equine/Prague/56 (H7N7) was in use in Croatia in 2004. In this study, we describe an outbreak of influenza at the Zagreb hippodrome in 2004 during which vaccinated and unvaccinated animals showed similar clinical signs. Antigenic drift between vaccine and outbreak strains is discussed as an explanation for vaccine failure.

2. Materials and methods

After clinical examination of horses on Zagreb hippodrome, nasal swabs and blood samples were taken from 11 horses showing clinical signs of acute respiratory diseases at the start of an outbreak on 2nd April 2004. Convalescent sera were taken from the same 11 horses 10 days later. For isolation of influenza virus, nasal swab extracts were inoculated into the allantoic cavity of 10-day-old embryonated eggs. Allantoic fluids were harvested after 3 days of incubation and checked for haemagglutinating (HA) activity. After three passages, the HA of allantoic fluid was $\geq$32 for inoculated extract from nasal swabs of five horses showing clinical signs at the start of the outbreak. Isolated virus stocks were lyophilised and sent to the Animal Health Trust (AHT), Newmarket, UK, for further characterization. Specific antibody levels to equine influenza virus were determined in sera sampled at the onset of the outbreak and 10 days later by HI and SRH assays. The HI assay was performed on microtitre plates according to standard procedures (OIE, 2000). SRH assay were carried out using sheep erythrocytes sensitised with virus as described previously (Wood et al., 1983). Viral RNA (vRNA) was extracted from allantoic fluid using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Both cDNA synthesis and PCR amplification of the HA1 gene were performed in a single tube using the One-Step RT-PCR with Platinum® *Taq* Kit (Invitrogen life technologies) as described previously (Borchers et al., 2005). The PCR amplifies HA1 products were purified using the QIAquick PCR purification Kit (Qiagen) according to the manufacturer's instructions. The purified PCR products were then sequenced using Big Dye 2 (Applied Biosystem) and analysed on a 3100 DNA Analyser for electrophoresis (Applied Biosystem). The nucleotide sequences were assembled and translated using the SEQMAN and

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EDITSEQ programs of the DNASIS package, respectively. Translated HA1 sequences of outbreak viruses and other used viruses were aligned using Clustal W (Higgins et al., 1996). Phylogenetic analyses were performed using the PHYLIP software package (Felsenstein, 1993). The dataset of HA1 sequences, including vaccine strains used on Zagreb hippodrome, strains used in vaccines in other regions of the world at the time of the outbreak, and used in phylogenetic analysis are available on the Los Alamos Influenza Sequence Database (Macken et al., 2001).

3. Results

In April 2004, an outbreak of equine influenza occurred at the Zagreb hippodrome. During clinical examination at the start of the outbreak 11 horses showed similar clinical signs of acute respiratory disease. During the next 2 days, 16 other horses showed similar clinical signs. The horses clinically examined at the start of the outbreak were of different gender, age and influenza vaccine history, from regularly vaccinated to non-vaccinated. None of these factors influenced the intensity of the clinical signs (Table 1). All vaccinated horses on the Zagreb hippodrome were vaccinated with vaccine containing strains A/equine/Miami/63 (H3N8), A/equine/Fontainebleau/79 (H3N8) and A/equine/Prague/56 (H7N7). Paired serum samples were used for diagnosis of equine influenza by serology using HI and SRH assays. All 11 horses showed significant increases in titre against subtype H3N8 viruses (A/equine/Newmarket/1/93 and A/equine/Newmarket/2/93) (Table 2) and none of the horses showed significant increase in titre against subtype H7N7 (A/equine/Prague/56). From these results, the outbreak strain was typed as subtype H3N8. Results of serology assays of the first (acute) sera samples confirmed differences in vaccine history. Three horses were seronegative and in the other eight pre-infection antibody levels to equine influenza strains A/equi/Newmarket/1/93 and A/equi/Newmarket/2/93 measured with HI were from 8 to 32, and with SRH assay from 18.5 mm^2 to 153.5 mm^2 (Table 2). After three passages in embryonated eggs, allantoic fluid from eggs inoculated with 8 of the 11 nasal swab extracts had HA activity. Five isolated equine influenza viruses had HA titres $\geq$32 and were further characterized. The results of the HA1 gene sequencing of the five outbreak isolates that were sequenced demonstrated only a single non-coding nucleotide substitution among them at nucleotide position 172. For that reason one translated amino acid sequence was used to compare outbreak strain with used and recent vaccine strains. Results showed 33 amino acid changes between outbreak strains and used vaccine strain A/equine/Miami/63. Fifteen changes occurred in four antigenic sites. Between A/equine/Fontainebleau/79 and the outbreak strain, 20 amino acids changes were observed, of which 9 were in 4 antigenic sites. In comparison with vaccine strains used in Europe at the time of the outbreak, there were 16 amino acids changes compared with the European lineage prototype strain A/equine/Newmarket/2/93 with nine changes distributed in all five antigenic sites, but only eight changes in comparison with the American lineage prototype strain

Table 1
Details of gender, ages, vaccine history and clinical signs recorded in horses on hippodrome Zagreb 2004

Horses with clinical signs at first visit			Vaccination history	Clinical signs		
Horse	Gender	Age (years)	Last vaccination	Fever (°C)	Cough	Nasal discharge
1	♂	7	Never	40.2	+	Mucoid
2	♀	10	<1 year	38.1	+	–
3	♂	10	>1 year	40.0	+	–
4	♂	7	<1 year	41.0	+	–
5	♂	6	>1 year	40.9	+	Serous
6	♀	8	>1 year	39.3	+	Serous
7	♂	13	<1 year	39.8	–	Serous
8	♂	6	<1 year	39.2	+	Mucoid
9	♂	7	>1 year	40.0	+	–
10	♂	7	Never	40.1	+	–
11	♀	4	Never	40.1	–	Serous

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Table 2
Antibody titre by HI and SRH assay using antigens A/equi/Newmarket/1/93 and A/equi/Newmarket/2/93 in acute and convalescent sera sampling from 11 horses with clinical signs at a first day of an outbreak.

	HI titre				SRH titre (mm^2)			
	Acute sera		Convalescent sera		Acute sera		Convalescent sera	
Horses	New/1/93	New/2/93	New/1/93	New/2/93	New/1/93	New/2/93	New/1/93	New/2/93
1	<4	<4	512	512	-	-	191,4	174,3
2	16	32	256	128	73.0	77.8	164,9	162,6
3	8	16	512	512	24.1	26.1	236,1	225,2
4	32	16	256	128	104.1	121.5	181,5	164,9
5	8	16	1024	512	27.1	39.5	261,6	255,8
6	8	16	512	256	60.8	65.3	201,5	176,7
7	32	32	512	512	146.8	153.5	222,5	211,9
8	32	16	512	512	93.2	94.9	198,9	191,4
9	16	8	512	512	18.5	28.2	214,5	204,1
10	<4	<4	512	256	-	-	222,5	193,9
11	<4	<4	512	512	-	-	233,4	217,1

Results for horses with clinical protective titre in acute sera is shaded gray.

A/equine/Newmarket/1/93 was observed of which only two were in antigenic site B and one in antigenic site C, while antigenic sites A, D and E were unchanged. Only four amino acid changes in the HA1 subunit protein were seen between the outbreak strain and a vaccine strain used in the USA A/equine/Kentucky/97 and all five antigenic sites were unchanged. In comparison with the most recently recommended vaccine strain, A/equine/South Africa/4/03, 2 amino acid changes was showed, but both on antigenic sites (B and E) (Table 3). The phylogenetic analyses confirmed that outbreak strain belonging to American lineage Florida sub-lineage of equine influenza viruses (Fig. 1). Results demonstrated that outbreak strain is evolutionary distinct from used vaccine strains, and most related to vaccine strains from the same sub-lineage A/equine/Kentucky/97 and A/equine/South Africa/03.

4. Discussion

The objective of our study was to investigate an outbreak of equine influenza at the Zagreb hippodrome in Croatia in 2004. Despite recommendations to update equine influenza vaccines with recent strains, in Croatia in 2004 vaccine that contained old strains, A/equine/Miami/63 (H3N8), A/equine/Fontainebleau/79 (H3N8) and A/equine/Prague/56 (H7N7), was still in use. Clinical signs in all animals examined were the same without influence of vaccine history which indicated complete vaccine failure without clinical protection (Table 1). Generally, in vaccinated animals disease is subclinical or with fewer clinical signs for a shorter time than in unvaccinated animals (Heldens et al., 2004). In our study, horses 4 and 7, with SRH antibody levels usually regarded as sufficient for clinical protection (Table 2), showed similar clinical signs to the unvaccinated horses (1, 10 and 11), which could be due to the effects of vaccine and challenge strain variation. The HA gene was targeted in the genetic characterization and phylogenetic analysis, because its mutation rate has been demonstrated to be greater than that of other viral genes and because it is one of the main targets of the humoral protective immune response (Paillot et al., 2006) and the location of five major antigenic sites on HA1 have been mapped (Wiley and Skehel, 1987). Analysis of amino acid sequences in antigenic sites between the Zagreb 2004 outbreak strain and the vaccine strains used showed changes in antigenic sites A–D (Table 3). At the time of the outbreak, the recommendation was to incorporate representative

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Table 3
Amino acid alignment sequences in antigenic regions of the HA1 of strain A/equi/Zagreb/04 compared with sequences of same regions in vaccine strains used on hippodrome Zagreb, and sequences of past and present representative strains

	Antigenic regions on HA1 subunit protein									
Viral strains	**A**		**B**		**C**			**D**		**E**
	132-146	155-163	186-199	48-55	273-278	170-174	201-218	242-248	78-83
Zagreb/04	QNGRSGACKRGSADS	TKSGNSYPT	SSNQEQTKLYIQES	MGKICNNS	PIDICV	NNKNF	RVTVSTKRSQQTIIPNIG	ILMINSN	VFQYEN
Miami/63	...G.S..R.....	.Q.ES.....	.T.N.....V.A.	T.....P	...T..	..D..	..... V.....	.....	.....
Fontainebleau/79	.....R.....	.....	.T.N.....V..L	I.....P	...T..	..N..	.....	.....	.....
Newmarket/1/93	.....	.....	....Q..E.....	I.....	.....	.....	.....	.....	.....
Newmarket/2/93	...G.....	I	...K.....	I.....	L..T..	.....	.....E.....V.....	.....	D.....
Kentucky/97	.....	.....	.....	.....	.....	.....	.....	.....	.....
South Africa/4/03	.....	....S....	.....	.....	.....	.....	.....	.....	A.....

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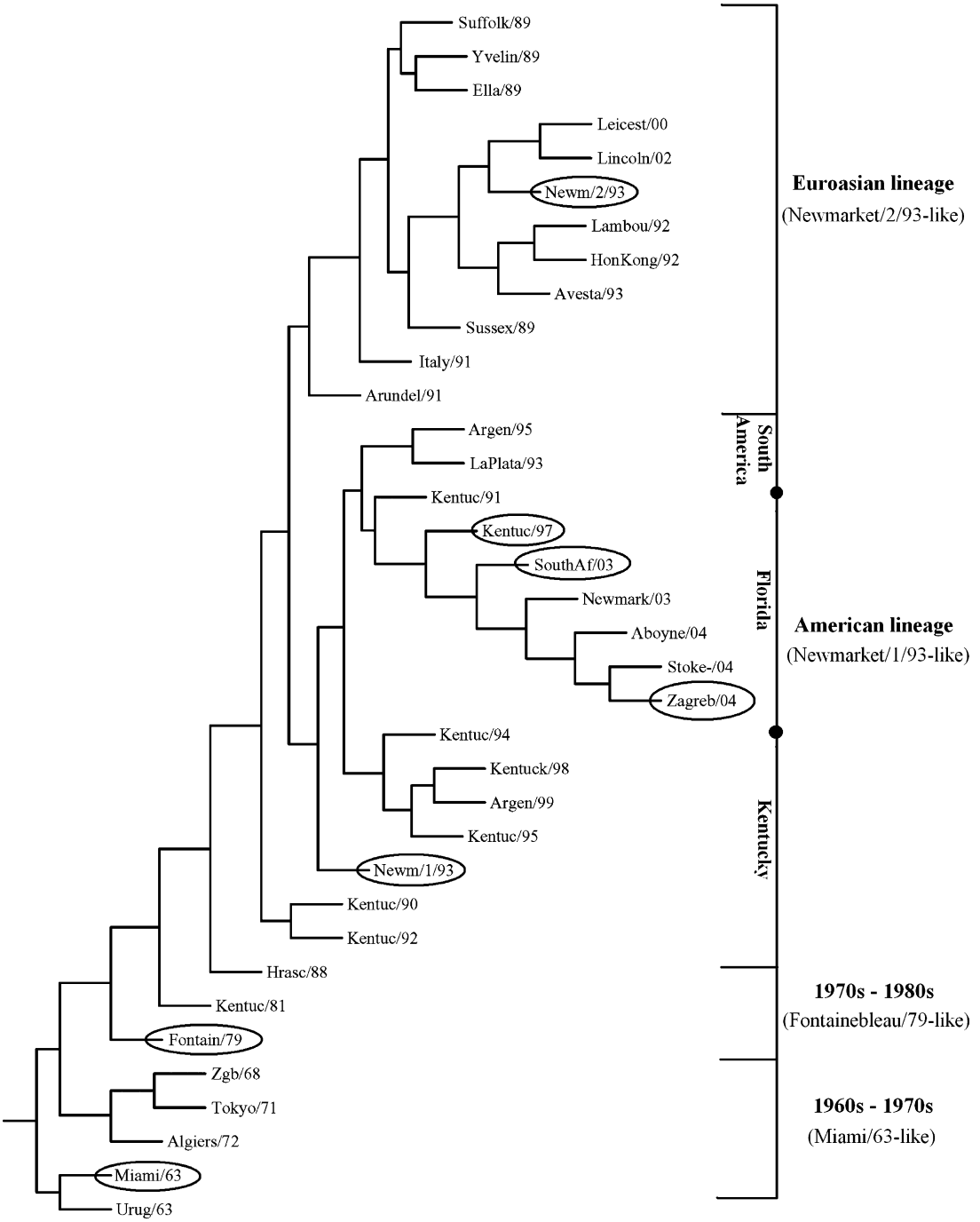

Fig. 1. Phylogenetic tree for the haemagglutinin (HA1) amino acid sequences of equine influenza viruses. Viral strain isolated during the Croatian outbreak in 2004 and strains compared within vaccine failure analysis are circled.

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strains of the American and European lineages in vaccines. Only three amino acid substitutions on antigenic sites were found between the outbreak strain and the American lineage prototype strain A/equine/Newmarket/1/93 included in most vaccines available in Europe at the time of the outbreak. Substitutions produced changes on antigenic sites B and C while antigenic sites A, D and E were unchanged. Amino acid sequences at antigenic sites were identical between the outbreak strain and A/equine/Kentucky/97, the vaccine strain used in the USA. Aligned with the new recommended prototype for the American lineage, A/equine/South Africa/4/03, the outbreak strain has only two amino acid substitutions, but both are on antigenic sites (B and E). Phylogenetic analysis confirmed significant evolutionary differences between A/equine/Zagreb/04 and used vaccine strains. Virus A/equine/Zagreb/04 belongs to American lineage sub-lineage Florida and it is evolutionarily closely related to vaccine strains from the same sub-lineage, A/equine/Kentucky/97 and A/equine/South Africa/4/04 (Fig. 1).

In conclusion, in this study we isolated equine influenza virus belonging to the Florida sub-lineage which caused an outbreak at the Zagreb hippodrome that affected vaccinated and unvaccinated animals regardless of vaccine history. Even horses with clinically protective SRH antibody levels showed the same clinical signs as unvaccinated animals due to vaccination with antigenic and evolutionary significant different vaccine strains in contrast to Expert Surveillance Panel recommendations. Use of heterologous vaccine strains may produce some degree of clinical protection but apparently not if challenge and vaccine strains have significantly different antigenic sites. Further surveillance of the equine population and updating of equine influenza vaccine strains in accordance with the recommendations of the Expert Surveillance Panel is necessary in Croatia to reduce the likelihood of further outbreaks as a result of vaccine failure.

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