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AN OUTBREAK OF EQUINE INFLUENZA (H3N8) IN CROATIA IN 2004 CAUSED BY SOUTH AMERICAN LINEAGE

INFLUENCA KONJA U HRVATSKOJ 2004. UZROKOVANA JUŽNOAMERIČKOM PODLINIJOM SEROTIPA A EQUI-2 (H3N8)

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Summary

Several epizootics of equine influenza have been reported in Croatia in the past. Both subtypes, A/equine-1 (H7N7) and A/equine-2 (H3N8) were isolated. An outbreak of equine influenza in 2004, in racehorses on Zagreb hippodrome is described. Both unvaccinated and vaccinated horses showed clinical signs of respiratory disease. By cross HI test and serological examinations of paired sera of apparently affected horses, equine influenza (H3N8) virus was detected. Antigenic and genetic analysis of isolated viruses revealed the occurrence of South American sublineage of equine-2 (H3N8) influenza virus. The results presented in this work demonstrated that the HA1 gene sequence of the 6 isolates that were sequenced had only a single, non-coding nucleotide substitution among them. When the amino acid sequences of the Croatian isolates were aligned with a virus isolated in the

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UK during a widespread outbreak in the spring of 2003 (Newmarket/5/03), only a single amino acid substitution in the signal sequence was seen.

Key words: Equine influenza; Serotype H3N8; South American sublineage

INTRODUCTION

Equine influenza is caused by two types of influenza A viruses with prototype strains A/eq/Prague/56 (H7N7) and A/eq/Miami/63 (H3N8) [1,2]. Outbreaks of equine influenza have been reported from many countries. However, until now Australia, New Zealand and Iceland have remained free from this disease [3]. Susceptible horses often develop non-specific signs of respiratory tract disease with sudden onset of an outbreak, very rapid spread, and harsh, dry and frequent cough. Fever, lethargy and anorexia are usually the first signs. Serious nasal discharge is frequently observed. In some cases fatalities can occur [3,4].

In the past several epidemics of equine influenza have been reported in Croatia. Both subtypes have been isolated [5-7]. Antibodies against H7N7 were detected in unvaccinated horses in Croatia suggesting that it continues to circulate in a subclinical form [3,8].

The antigenic drift of H3N8 strains from prototype strain Miami/63 has been frequently demonstrated and outbreaks of H3N8 virus often occur [3,9]. Daly et al. [10] revealed that two genetically and antigenically lineages of equine influenza H3N8 virus emerged during the mid-1980s. The American lineage is widespread, and Eurasian lineage seems to be mainly confined to Europe.[11] These two lineages have continued to cocirculate, particularly in European countries. However, Lai et al. [11] reported that the American lineage had further diverged as a variant of the American lineage has arisen in South America (the South American sublineage). This diverged evolution of equine-2 influenza virus has significant impact in the selection of vaccine strains for influenza viruses [11].

In this paper some characteristics of an outbreak of equine influenza in Croatia in 2004 are described. All isolates from this outbreak belonged to the South American branch of the American lineage.

MATERIALS AND METHODS

Sampling

On 2nd April 2004, the local practitioner observed clinical signs of lethargy, anorexia, frequent coughing and nasal discharge in 11 racehorses on Zagreb hippodrome. Clinical signs of acute respiratory disease developed over the next 4 days in these horses.

On 3rd April 9 new cases were reported, next day 4 new cases, and finally, on 5th April, 4 new cases. Disease occurred in vaccinated and unvaccinated horses.

The first of paired serum samples were taken from 11 horses with clinical signs of respiratory disease on 2nd April. At the same time nasal swabs were taken from the same 11 horses. After sampling, the swabs were immediately placed in virus transport medium (HMEM/antibiotics/2% FBS). After centrifugation of the swab, 2 mL of transport medium was stored at –70 °C for virus isolation.

Serology

Antibody responses to equine influenza were determined by the haemagglutination inhibition (HI) test. The HI test was performed on microtitre plates according to standard procedures. Before the assay, the sera were inactivated at 56 °C for 60 min, and absorbed overnight at 4 °C with chicken red blood cells. Antigens used were equine influenza viruses A/eq/Zagreb/73 (H7N7), A/eq/Zagreb/68 (H3N8), and A/eq/Hraščina/88 (H3N8).

Virus isolation

For isolation of influenza virus, nasal swab extracts were inoculated into the allantoic cavity of 10-day-old embryonated hens' eggs. For each sample, 5 eggs were inoculated with 0.2 mL of swab extract per egg, and incubated at 37 °C. Allantoic fluids were harvested after 3 days of incubation and checked for haemagglutinating (HA) activity. Virus isolates were sent to the Animal Health Trust (AHT), Newmarket, UK an Office International des Epizooties international reference centre for equine influenza, for further analysis and gene sequencing.

Antigenic characterization of equine influenza virus isolates

Haemagglutination inhibition (HI) assays were carried out per Daly et al. [10] against a panel of 33 post infection ferret antisera treated with periodate and heat to remove non-specific inhibitors of agglutination. In addition, the viruses were tested against a monoclonal antibody (MAb) raised against the H3N8 American lineage prototype strain Newmarket/1/93.

Genetic characterization of equine influenza virus isolates

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). A mix containing 1x

final concentration Reaction Mix, 10 mL of extracted vRNA, 0.2 mM each of sense (5'-AGCAAAAGCAGGGGATATTTCTG-3') and anti-sense (5'-GCTATTGCTCCAAAGATTC-3') primers, 1 mL RT/Platinum *Taq* mix and 12 mL of water was added to 10 mL of template RNA. The following thermal cycler program was run: 50 minutes at 50 °C, 2 minutes at 94 °C, followed by 39 cycles of 94 °C for 15 seconds, 50 °C for 30 seconds and 70 °C for 1 minute, and finally 1 minute at 70 °C. The PCR amplified HA1 products were purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. The purified PCR products were sequenced using 2 mL DNA, 1.6 pmol forward or reverse primers, 2 mL Big Dye Terminator mix, 2 mL dilution buffer (400 mM Tris pH9.0, 10 mM $MgCl_2$) and deionised water in a volume of 10 mL. Reactions were performed in 96-well PCR plates (ABgene) for cycle sequencing with the following conditions; 96°C for 3 minutes, then 25 cycles of 96°C for 30s, 50°C for 15s and 60°C for 4 min. Sequencing reactions were precipitated by addition of 1 mL of 3M NaAc pH 4.6 and 25 mL of ice-cold 100% EtOH. Samples were mixed and incubated at room temperature for 15 min before centrifugation at 4000 rpm for 30 min in an Eppendorf 5804 benchtop centrifuge. Plates were inverted and centrifuged for 2 min at 250 rpm. Pellets were washed by adding 125 mL of 70% EtOH and centrifuging at 4000 rpm for 10 min. Plates were incubated at 37 °C for 30 min to dry and 10 mL Hi-Di formamide was added. Plates were heated at 95 °C for 1 min and transferred to ice for 2 min before being placed

Table 1. Serological results for influenza diagnosis in tested horses with clinical respiratory signs

Horse No.	Reciprocal HI titre					
	A/eq/Zagreb/73 (H7N7)		A/eq/Zagreb/68 (H3N8)		A/eq/Hraščina/88 (H3N8)	
	a	b	a	b	a	b
1⁻	16	32	<4	512	8	512
2⁺	32	64	32	512	16	128
3⁺	32	64	16	512	8	512
4⁺	32	64	16	128	32	128
5⁻	8	16	8	512	4	512
6⁺	16	32	32	512	<4	512
7⁺	16	32	32	512	32	512
8⁺	16	32	32	512	32	512
9*	8	4	16	512	8	512
10*	8	16	8	64	<4	128
11⁻	16	32	8	512	<4	512

⁺ regularly vaccinated
⁻ unvaccinated
* vaccinated more than one year ago
a = acute sera, b = convalescent sera

onto a 3100 DNA Analyser for electrophoresis. The nucleotide sequences were assembled and translated using the SEQMAN and EDITSEQ programs of the DNASIS package, respectively. A representative sequence has been submitted to the Influenza Sequence Database (http://www.flu.lanl.gov/) [12].

RESULTS

Clinical signs observed were rather mild in all observed horses. All horses showed a rise in body temperature and nasal discharge for 3-4 days. Frequent harsh, dry coughing and mucous nasal discharge were found. We treated with antibiotics all horses with temperature higher than 40 °C. Clinical signs disappeared 5-7 days after treatment start.

Table 2. Haemagglutination titre of allantoic harvests

Horse No.	HA titre (reciprocal)		
	I passage	II passage	III passage
1	32	32	64
2	0	0	0
3	16	32	64
4	0	0	0
5	4	16	32
6	16	16	16
7	32	32	>64
8	0	8	16
9	4	32	32
10	0	0	0
11	16	32	>64

Serological findings are given in Table 1. No seroconversion was found to the A/eq/Zagreb/73 (H7N7) antigen in the tested sera. The HI titres to A/eq/Zagreb/68 (H3N8) and A/eq/Hraščina/88 (H3N8) in all tested samples showed a significant (fourfold or greater) increase between the acute and convalescent sera indicating recent infection with an H3N8 subtype virus.

Table 2 shows the results of haemagglutination of the eleven tested specimens in three passages. No virus was isolated from specimens 2, 4, and 10 after 3 passages. Haemagglutination titer increased with each passage, except for specimen 6, where titre through the 3 passages was the same. The 8 virus isolates obtained, Zagreb/1/04 (Z/1/04), Zagreb/3/04 (Z/3/04), Zagreb/6/04 (Z/6/04), Zagreb/7/04 (Z/7/04), and Zagreb/11/04 (Z/11/04), were sent to the AHT for antigenic and genetic characterization.

The HI reactivity patterns of Croatian A/eq/Zagreb/04 (H3N8) isolates as compared with some others isolates are given in Table 3. Highest HI titres were obtained with

Table 3. Reactivity patterns of A/eq/Zagreb/04 (H3N8) influenza virus isolates by HI panel of ferret antisera

Ferret antiserum raised to	Reciprocal HI titre						
	Homol.	Z/1/04	Z/3/04	Z/6/04	Z/7/04	Z/11/04	GMT
Pre-1989 prototype strains							
Miami/63	32	11	4	4	8	4	5*
Fontainebleau/79	91	512	128	256	256	256	256
312Kentucky/81	25	48	32	16	32	32	31
European-lineage prototype strains							
Suffolk/89	11	8	4	4	8	8	7
Sussex/89	256	24	32	16	32	32	28*
Newmarket/2/93	222	8	4	4	4	4	37*
American-lineage prototype strains							
Newmarket/1/93	128	181	64	64	128	64	113
Kentucky/98	362	128	64	64	128	128	108
Recent isolates							
Argentina/99	157	384	256	128	256	256	247
Kentucky/99	128	256	128	128	128	128	139
Leicestershire/1/00	128	91	64	32	32	64	56
Lincoln/1/02	256	32	16	8	32	32	25*
Newmarket/5/03	256	384	128	128	128	128	147
Benelux/19/03	512	48	16	16	32	32	28*
South Africa/4/03	431	91	64	64	128	128	103*
MAb	>1024	4	4	4	4	4	4

* Geometric mean HI titre of the 5 Zagreb/04 isolates more than 4-fold lower than homologous titre for ferret serum

Newmarket/5/03, Argentina/99, Kentucky/99, and Kentucky/97. There also appears to be an epitope shared with Fontainebleau/79.

HA1 gene sequence analysis of 6 of the A/eq/Zagreb/04 (H3N8) isolates showed a single, non-coding, nucleotide substitution among them (Table 4). When the amino acid sequences of the Zagreb/04 isolates were aligned with a virus isolated in the UK during a widespread outbreak in the spring of 2003 (Newmarket/5/03), only a single amino acid substitution in the signal peptide (L-9F) was seen (Table 4).

DISCUSSION

The objective of our study was to isolate and characterize influenza isolates causing a respiratory disease outbreak in racehorses on Zagreb hippodrome in 2004.

Table 4. Nucleotide and amino acid substitutions in the HA1 sequences of 6 A/eq/Zagreb/04 (H3N8) isolates compared with A/eq/Newmarket/5/03

ISOLATE	Nucleotide (amino acid) location			
	21 (-9)	120 (35)	171 (52)	231 (72)
Newmarket/5/03	G (L)	A (E)	G (C)	C (G)
Zagreb/1/04	T (F)	C (E)	A (C)	T (G)
Zagreb/3/04	T (F)	C (E)	G (C)	T (G)
Zagreb/5/04	T (F)	C (E)	G (C)	T (G)
Zagreb/6/04	T (F)	C (E)	G (C)	T (G)
Zagreb/7/04	T (F)	C (E)	G (C)	T (G)
Zagreb/11/04	T (F)	C (E)	G (C)	T (G)

Daly et al. (1996) [10] demonstrate, that since around 1989 a divergent evolution of American and European isolated of equine-2 influenza virus has occurred resulting in two antigenically and genetically distinct lineages. Subsequently, there has been further divergence of the American lineage resulting in a South American branch or sublineage [11]. The Zagreb isolates were characterized as belonging to the South American branch of the American lineage of H3N8 viruses. In haemagglutination inhibition assays with ferret antisera, low titres were obtained with sera raised to European lineage isolates, and the highest titres were obtained against sera raised to strains belonging to the South American branch. There also appeared to be an epitope shared with Fontainebleau/79. The results presented in this paper demonstrated that the HA1 gene sequence of the 6 isolates that were sequenced had only a single, non-coding nucleotide substitution among them. When the amino acid sequences of the Croatian isolates were aligned with a virus isolated in the UK during a widespread outbreak in the spring of 2003 (Newmarket/5/03), only a single amino acid substitution in the signal sequence was seen. The same substitution was also observed in a virus isolated in the UK in 2004 (Daly, unpublished observation). It is thought that the equine influenza strain responsible for the outbreak of equine influenza in Newmarket in March and April of 2003 was brought into the UK with the importation of an infected horse as the HA1 sequence for this virus was identical to one isolated in the USA in 2002. The virus spread to other European countries including Ireland, Italy and France in the following months. That the Croatian viruses isolated in 2004 shared an amino acid change with a UK isolate from 2004 suggests that the source of the outbreak at the Zagreb hippodrome was an infected horse from the UK. Viruses isolated during an outbreak in South Africa in December 2003 were found to be identical to viruses circulating in America during 2003. These viruses had acquired further mutations in the HA1 that distinguished them from the viruses isolated in Europe during 2003 and 2004.

It has been demonstrated that, for optimal efficacy, equine influenza vaccines should contain strains representative of currently co-circulating lineages of H3N8 equine influenza virus in the field. Following the 1995 recommendations of World Health Organization and Office International des Epizooties (OIE) Expert Surveillance Panel, vaccines including a representative of the European lineage, such as Suffolk/89 or Newmarket/2/93, and a representative of the American lineage such as Newmarket/1/93 or Kentucky/94 (OIE, 1996) were in use at the time of the 2004 outbreak in Zagreb. Although the South American variants arising in the UK in 2003 caused widespread outbreaks even in heavily vaccinated populations, the viruses did not appear to be significantly different from the prototype American lineage vaccine strain (Newmarket/1/93). In the meantime, the additional 2 amino acid substitutions that arose in the HA1 viruses circulating in North America during 2003 did give rise to significant antigenic differences. It was therefore recommended, in 2004, that South Africa/4/03, or a very closely related strain, replace the American lineage vaccine strain.

In conclusion, in this study we detected virus belonging to the South American sublineage of equine influenza virus for the first time in Croatia. Both vaccinated and unvaccinated animals were affected. Further surveillance of equine population grazed in the fields is needed.

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Sažetak

U prošlosti je zabilježeno nekoliko epizootija influence konja u Hrvatskoj. Izdvojena su bila oba serotipa virusa: A-1 equi (H7N7) i A-2 equi (H3N8). U radu je opisana pojava influence konja na zagrebačkom hipodromu u 2004. godini. Kliničke znakove dišne bolesti pokazivali su cijepljeni kao i necijepljeni konji protiv influence. Unakrižnom reakcijom inhibicije hemaglutinacije te serološkom pretragom parnih seruma oboljelih konja dokazano je da je bolest uzrokovao serotip H3N8. Antigenska i genetička analiza pokazala je da svi izdvojeni izolati virusa pripadaju južnoameričkoj podliniji serotipa H3N8. U svih šest analiziranih izolata ustanovljena je supstitucija jednog nekodirajućeg nukleotida u genu za hemaglutinin HA1. Kad je uspoređen slijed aminokiselina hrvatskih izolata s onima izdvojenima u proljeće 2003. u Engleskoj (Newmarket/5/03) dokazana je supstitucija jedne aminokiseline u signalnom slijedu.

Ključne riječi: Influenca konja; Serotip H3N8; Južnoamerička podlinija