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Short communication

Isolation and characterization of the equine influenza virus causing the 2006 outbreak in Chile

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ABSTRACT

The equine influenza virus is the causal agent of influenza in horses. In July 2006, horses from various regions of Chile presented fever, serious nasal discharge, dry cough, anorexia and depression. Here we describe the isolation and characterization of the virus responsible for this outbreak. The virus was identified as equine influenza virus H3N8, since haemagglutination was inhibited by an anti-A/equi/1/H3N8 serum, but not by an anti-A/equi/1/H7N7 serum. The isolate was named A/equi/2/Lonquén/06 (H3N8). In addition, we describe the isolation and sequencing of the haemagglutinin, neuraminidase and nucleoprotein genes of this new isolate. Sequence alignments show important differences with the Santiago/85 isolate and a closer relation to North American isolates, especially with the Florida lineage, and to Argentina isolates from 1990s.

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1. Introduction

Equine influenza (EI) is an acute contagious disease that affects the upper airways in equids and it occurs generally in epidemic outbursts. It is caused by the equine influenza virus (EIV) type A, a member of the *Orthomyxoviridae* family. Two different subtypes of equine influenza virus, H7N7 and H3N8, have been associated with the disease. Based on minor antigenic differences, two lineages have been described for H3N8; the European lineage and the American lineage (Daly et al., 1996). Strains within the American lineage further diverged into three sublineages, South American, Florida and Kentucky (Lai et al., 2004). The subtype H7N7 is apparently extinct, since the last outburst was described in 1979. In contrast, subtype H3N8 prevails and appears as periodic outbursts. The EIV is ubiquitously distributed through nature and the only countries that are still free of this virus are Iceland and New Zealand (Murphy et al., 1999).

In Chile, the first outburst of equine influenza was described in June 1963 (Fuschlocher et al., 1963), the same year that the subtype H3N8 was isolated from an outburst that affected several states in the United States (Waddell et al., 1963). In the summer of 1977 there was an outburst from which the EIV was isolated for the first time in Chile, corresponding to the subtype A/equi/1/Santiago/77(H7N7) (Casanova et al., 1977). Afterwards, in mid-December of 1985, a new outburst appeared, from which the subtype A/equi/2/Santiago/85(H3N8) was isolated (Berríos et al., 1986). Another outburst was reported in Chile in March 1992, and the isolate was identified as virus A/equi/2/Quillota, Chile/92(H3N8) (Celedón et al., 1992). Müller et al. (2005) demonstrated that the A/equi/2/Santiago/85(H3N8) isolate belongs to the American lineage and has significant differences when compared to other European and American isolates.

In June 2006, clinical signs corresponding to equine influenza were found in animals from different regions in the country but no mortalities were reported. Here we describe the characterization of the viral isolate, based on

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the sequences of haemagglutinin, neuraminidase and nucleoprotein.

2. Materials and methods

Nasal swabs from two horses with clinical signs of equine influenza were collected in tubes containing 2 ml physiological saline solution with antibiotics. Viral isolation was performed in embryonated chicken eggs. Detection was carried out using a haemagglutination test and the isolate was identified by a haemagglutination inhibition test.

For virus isolation, collected samples were centrifuged at 1500 × *g* for 15 min and 100 μl of supernatant was inoculated on each of ten embryonated chicken eggs (10 days of development) through the allantoic cavity. After 5 days at 37 °C, eggs were cooled to 4 °C and allantoic fluid was collected, subjected to haemagglutination test and used to re-inoculate embryonated chicken eggs, repeating the process up to 5 times (OIE, 2000). The haemagglutination and haemagglutination inhibition test were performed by standard procedures (OIE, 2000).

Viral RNA was isolated from allantoic fluid from 5th egg passage, according to the protocol described by Chomczynski (1993). This RNA was used to amplify and isolate genes by RT-PCR, using random hexanucleotides (Sambrook and Russell, 2001).

The cDNA was amplified by PCR using primers designed according to the conserved regions described by Müller et al. (2005) and cloned in the pCR2.1 plasmid (Invitrogen).

The genes of interest were sequenced using the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) (Sanger et al., 1977). The sequences are available in Genbank under EU926629 for nucleoprotein, EU926630 for neuraminidase and EU926631 for haemagglutinin.

Sequence alignments were carried out using BLAST of NCBI (http://www.ncbi.nlm.nih.gov/BLAST) and Clustal X1.8 software (Thompson et al., 1997). MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) was used to perform the Bayesian phylogenetic analyses and MEGA version 4 (Tamura et al., 2007) was used to perform the neighbor-joining method. Models of sequence evolution were selected using MrModeltest 2.2 (Nylander, 2004). Data sets were analyzed using the models HKY + I + G. The trees obtained were imported into Treeview (Page, 1996), and a majority rule consensus tree was computed after discarding the first 25% of the trees to account for "burnin", which corresponds to the set of trees obtained before the likelihood scores plateau over an optimal value. Support for clades given by posterior probabilities was thus represented by the percentage obtained of the summary tree.

In addition, a neighbor joining (NJ) bootstrap analysis with 1000 replicates was done using MEGA and a Maximum Composite Likelihood model, with different rates for transitions and transversion, patterns among lineages and rates among sites, which were estimated from the data.

3. Results and discussion

The 2006 outbreak affected mainly young animals (1–5 years old) without distinction of gender or race. Clinical signs appeared during 5–7 days, declining afterwards in intensity and frequency and giving way to complete remission at 10–12 days. During the present outbreak, animals under vaccination calendars that included complete or incomplete equine influenza vaccine were also affected, but symptoms were more moderate in intensity and duration. One group of horses had been vaccinated a year early with a preparation containing strains Prague/56 (H7N7), Lexington/63 (H3N8) and Kentucky/81 (H3N8). Another group was vaccinated a year early with Prague/56 (H7N7), Newmarket/1/93 (H3N8) and Newmarket/2/93 (H3N8). Since the symptoms were present in horses vaccinated with H7N7 and H3N8 strains, it was of interest to characterize the EIV of the 2006 outbreak.

During viral isolation, allantoic fluid collected from the 3rd and 5th passages presented agglutination titres of 16 and 64, respectively. The haemagglutination inhibition test with the anti-H3N8 serum showed a haemagglutination inhibition titre of 128, demonstrating that the isolate corresponds to EIV H3N8 and was named A/equi/2/Lonquén, Chile/06 (H3N8). Accordingly, no inhibition was observed with the anti-H7N7 serum.

Although the recent isolate is similar to the subtype H3N8 of the 1985 and 1992 isolates, it was of interest to know whether there were minor antigenic differences to be considered in the elaboration of future vaccines for the Chilean equine population.

To determine these antigenic differences, nucleoprotein, neuraminidase and haemagglutinin genes were isolated, cloned and sequenced and compared to the sequences obtained from the A/equi/2/Santiago 85 isolate (Müller et al., 2005) (the only Chilean isolate with sequence data available) as well as those from other isolates (Table 1).

Nucleoprotein is an internal protein that does not affect the selection ability of the virus, but allows its classification among influenza groups. When the amino acid sequence is compared to the corresponding sequence from the Santiago 85 isolate we found 11 differences with 97% identity. This data indicates a mutation rate of approximately 0.5 events per year, which is close to the rate estimated previously by comparison to all the published sequences described in the literature (Müller et al., 2005). Comparison of 2002 and 2003 isolates with year 2000 sequences of nucleoproteins from the ISD database (Macken et al., 2001) shows differences in 5 amino acidic residues.

Neuraminidase is a membrane protein involved in the liberation of newly synthesized viral particles from infected cells and is one of the proteins responsible for virally induced distraction of the immune system. When compared to the corresponding sequence from the Santiago 85 isolate, both have the 7 putative N-glycosylation sites and the 17 cysteine residues that are highly conserved in neuraminidase subtypes. At the amino acid level there are 16 differences with a 97% similarity. This indicates a mutation rate of 0.76

Table 1
Amino acid variation in relevant antigenic sites of haemagglutinin H3 with the most representative recent isolates from each decade including those chosen for vaccines.

	Site A	Site B	Site C			Site D		Site E	
Isolate	147-161	202-214	67-70	288-293	185-189	222-230	256-261	59-63	93-99
Lo06	QNGRSGACKRGSADS	SNQKQTELYIQES	CNNS	PIDICV	NNKNF	KRSQQTIVP	DILMIN	QSSSI	VFQYENW
Ab05		..KE..K		I..T		E.....VI	.V	E.I.M	
Ba05		...E..K				......MI		..I.M	
La05		...E..K				......MI	...T	..I.M	
Wa05		...E..K				E.....V		..I.M	
Au04	......S	..EE..K				I		..I	
Ca03		...E..K				I		..I.T	
Oh03		...E..K				I		..I.M	A
Be02		..KE..K		...T		E.....VI	...T	..I	
Ke02		...E..K				I		..I.M	
Ch01		..KE..K		L		E.....VI	.V	..I	
Le00	...S	..KE..K		L..T		E.....VI	.V	..I.M	
Ar99		...E..K				I		..I	
Ar97		...Q				I		..I	
Ne93/2	...G	..KE..K		L..T		E.....VI		..I	D
Ne93/1		...Q				I		..I	
Su89		..KE..K		...T		E.....VI	...T	..I	
Sa85		..NE..N	...P	...T	..N	...R...I		..I	
Fo76	........R	T.NE..K..V..L	...P	...T	..N	I		..T	
Mi63	...G.S..R	T.NE..K..V.A	...P	...T	..D	I	.V	..T	

The following abbreviations do not appear in the text: Lo06: Lonquén 2006; Ab05: Aboyne 2005; Ba05: Bari 2005; La05: Lanark 2005; Wa05: Wales 2005; Au04: Arundel 2004; Ca03: California 2003; Oh03: Ohio 2003; Be02: Berlín 2002; Ke02: Kentucky 2002; Ch01: Cheltenham 2001; Le00: Leicestershire 2000; Ar99: Argentina 1999; Ar97: Argentina 1997; Ne93/2: New Market 1993 isolate 2; Ne93/1: New Market 1993 isolate 1; Su89: Sussex 1989; Sa85: Santiago 1985; Fo76: Fontainebleau 1976; Mi63: Miami 1963.

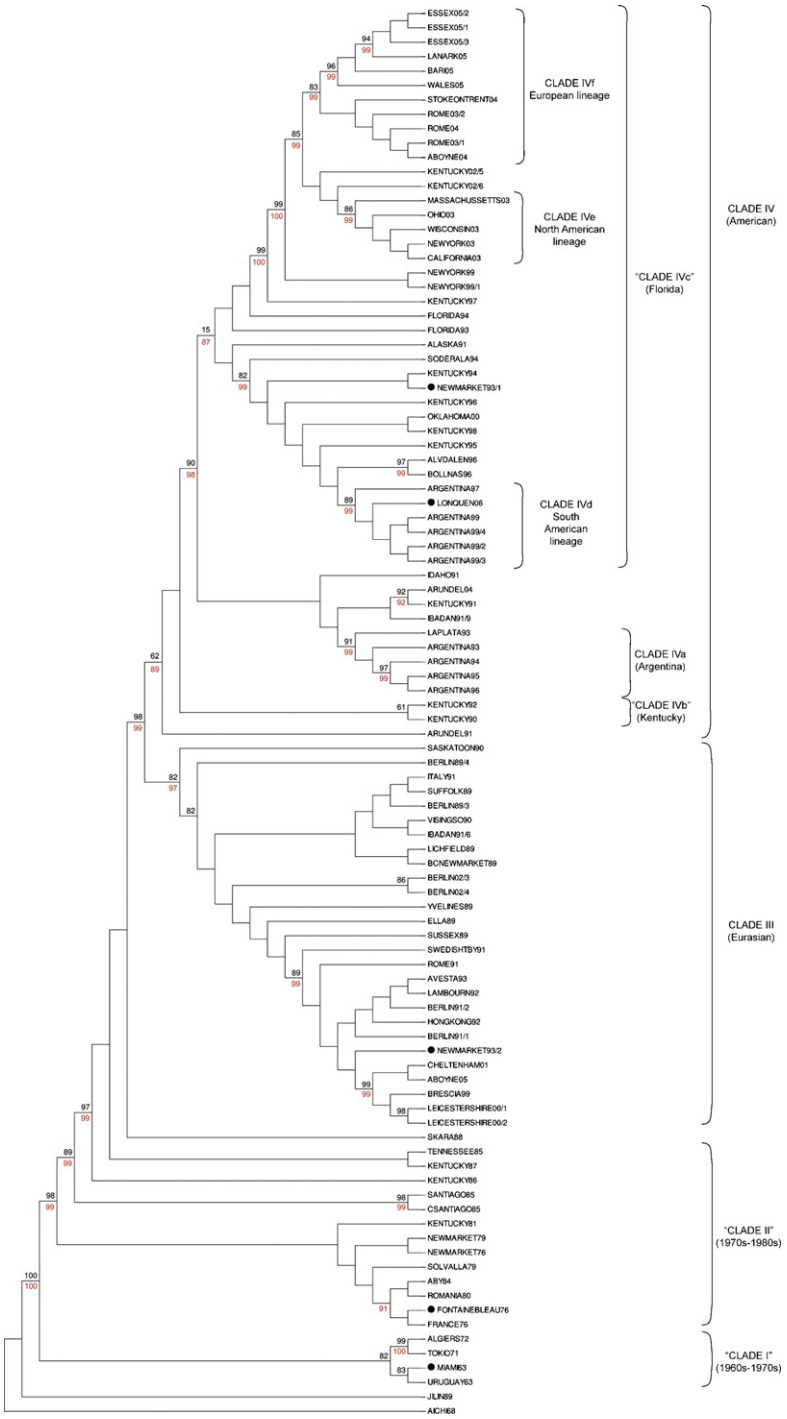

Fig. 1. Neighbor joining phylogenetic tree representing evolutionary relations among isolates of the equine influenza virus. Analysis of the HA nucleotide sequence of the H3N8 subtype ●: H3N8 prototypes. Clades I to IV and lineages were designated by Lai et al. (2001). Subclades IVa–IVc was designated by Lai et al. (2004). Subclades IVd–IVf designated in this paper correspond of isolate of the 2000 decade. Bootstrap percentages higher than 70% and posterior probabilities above 90% are marked above (in black) and below (in red) branches, respectively. The clades, between quotation marks, correspond to the previous study, which, in this study, do not represent a monophyletic group. The tree was rooted to the A/Aichi/2/1968 (H3N2) isolate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

substitutions per year. This result is in agreement with that calculated previously by comparison of all the sequences available in databases (Müller et al., 2005). Comparison of the amino acid sequence of the Lonquén 06 isolate neuraminidase with 6 ISD database sequences from 2000 and after, shows 11 differences with the Kentucky 02 isolate and 13 differences with the remaining five isolates.

Haemagglutinin is a membrane glycoprotein, responsible for the entrance of the virus into the host cell and is directly involved in the immunological evasion mechanism of the virus. Of these sequences, 77 nucleotides and 25 amino acids of the Lonquén 06 isolate are different from the sequence of the Santiago 85 isolate. Four amino acid differences correspond to changes unique for the Lonquén 06 isolate (residues 21, 93, 243 and 544); four of the differences with Santiago 85 appear in isolates from 1989 and after (residues 70, 187, 275 and 325); three differences appear in all of the analyzed isolates, except in Santiago 85 (residues 108, 225 and 238); five differences are found in the 1997 and 1999 Argentina isolates (residues 107, 119, 204, 208 and 229). The other 9 amino acids that differ from the Santiago 85 isolate are the same as found in isolates such as Kentucky 95, Oklahoma 00, Arundel 04, New York 99, etc. Both sequences have the same number of cysteine residues but Lonquén 06 has seven possible glycosylation sites in the HA1 polypeptide, whereas Santiago 85 has only six.

Comparison of the amino acid sequences of the relevant antigenic sites of Lonquén 06 (Wiley et al., 1981), with the sequence of the same site of the most representative isolates from each decade including those chosen for vaccines, shows several differences. In the antigenic site A (residues 147–161), there is little variation; in site B (residues 202–214) there are two or three differences; in antigenic site C (residues 67–70 and 288–293) there are two variations; in site D (residues 186–189; 222–230 and 256–261) there are one, two or three differences and in antigenic site E (residues 59–63 and 94–99) there is one or two differences (Table 1). The least number of differences is with the isolates New Market 93/1, main representative of the American lineage, and the Argentina 97 and Argentina 99 isolates, displaying 3 and 4 amino acid changes respectively, which would be enough to produce a significant antigenic drift (Wilson and Cox, 1990) which may partly explain the 2006 outbreak.

The phylogenetic tree constructed from the HA nucleotide sequences from 97 isolates, obtained from the ISD database is shown in Fig. 1. Here we show the clades that group together the different lineages, according to Lai et al. (2001). We found that the clades I and II are still hypothetical since they are not statistically supported. However clades III and IV are statistically significant.

According to our analysis, the subclassification of the American lineage in three sublineages (Lai et al., 2004; Martella et al., 2007) is also not statistically supported. The only statistically significant sublineage is the IVa-Argentina sublineage (formed by Argentine and La Plata isolates), which shows 91% of bootstraping and 99% posterior probability (Fig. 1).

Nevertheless, we can observe the formation of other statistically supported clades when analyzing the most recent isolates, which we shall designate as clade IVd to continue with the same nomenclature, formed by the Argentine isolate plus ours (South American lineage), and nested within the 1990s North American isolates. We can also observe the clade IVe, consisting of the 2003 North American isolates, clade IVf with 2003 and 2005 European isolates, formed a new geographical classification (Fig. 1).

In accordance with the above, we can conclude that the isolate under study, the Lonquén 2006, is included in the American lineage, nested within a well supported clade formed by the isolates from Argentina 97 and 99, as a sister group of the subclade formed by four 1999 Argentine isolates. Therefore, we can establish a biogeographic hypothesis that the Lonquén 2006 isolates came from Argentina.

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