Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

SHORT COMMUNICATION

Isolation and Characterization of Equine Influenza Viruses (H3N8) from China, 2010~2011

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ARTICLE HISTORY

Received: February 22, 2012
Revised: June 20, 2012
Accepted: August 19, 2012

Key words:
Equine influenza
Genetic analysis
Isolation

ABSTRACT

Two equine influenza virus (EIV) strains were isolated during two restricted outbreaks from Heilongjiang Province, China in 2010 and 2011. Phylogenetic analysis of HA1 (hemagglutinin 1) gene revealed that the isolates belonged to Florida 2 sublineage of American lineage. Further analysis of the putative antigenic sites located in HA1 subunit protein revealed each isolate had a unique amino acid change. Analysis of antigenic sites between Chinese EIV and vaccine strains indicated equine influenza (EI) vaccines containing Richmond/1/07-like antigen seemed to have an optimum effect in China. Meanwhile, the Ohio/03 vaccine strain contained in updated ProteqFlu had the most closely genetically relationship with recent EIV isolates in China. China has not its own commercially available EI vaccine and most horses are still unvaccinated. Therefore, to monitor antigenic variation of circulating EIVs and give considerable suggestions on selection of vaccine candidate plays an important role in preventing and controlling EIV in China.

To Cite This Article: Lu G, J Chen, W Guo, T Qi, L Zhao, H Li, Y Ji, Z Wang, C Liu, S Zhao and W Xiang, 2013. Isolation and characterization of equine influenza viruses (H3N8) from China, 2010~2011. Pak Vet J, 33(2): 238-240.

INTRODUCTION

Equine influenza virus (EIV), belonging to the *orthomyxoviridae* family, is generally associated with severe acute respiratory disease in horses. Until now, two subtypes of EIV, H7N7 and H3N8, are responsible as notifiable horse disease of EI listed by the World Organization for Animal Health (OIE). H7N7 subtype EIV has not been isolated for nearly 30 years in horse populations all over the world. Meanwhile, antigenic drift has given rise to the diversity of H3N8 subtype EIV with a worldwide distribution. The H3N8 virus diverged into two antigenically and genetically different evolutionary lineages since 1986: the American and the European lineage (Barbic *et al.*, 2009). And the American lineage further evolved into the Kentucky, South American and Florida sublineage clades 1 and 2 (Damiani *et al.*, 2009; Gildea *et al.*, 2011b; Motoshima *et al.*, 2011).

Between 2007 and 2008, a serious outbreak of EI occurred in China. And the epizootic mainly occurred in Northern China, such as Heilongjiang, Liaoning, Inner Mongolia, Xinjiang, and Gansu, and only one was isolated in Hubei, in southern China. The virus responsible for the epizootic event is identified as Florida sublineage clade 2 of H3N8 subtype EIV (Qi *et al.*, 2010). Moreover, during this period, the majority of EIVs responsible for the large outbreaks in Asia belonged to the Florida sublineage, such as in Japan (Florida 1), Mongolia (Florida 2) and Australia (Florida 1).

Vaccination can still be regarded as the most effective method for prevention of EI, though outbreaks of EI continue to infect some horse populations that have the extensive use of vaccines (Rozek *et al.*, 2009). Traditional vaccine strategies against influenza have focused on inducing antibody responses against the viral surface glycoproteins, particularly the hemagglutinin (HA). Whether protection induced by one virus strain is effective against another relies on the antigenic differences between them (Mumford, 1992). Therefore, to monitor the antigenic variation of HA and other viral proteins of new emerging virus strains is important for OIE to make decisions for the selection of appropriate vaccine strains.

§Contributed equally to this study

MATERIALS AND METHODS

Sample, virus isolation and sequencing: Two restricted outbreaks in two different herds of Heilongjiang Province in China were observed in 2010 and 2011. The infected horses presented clinical sighs of cough, high fever, serious nasal discharge and dyspnea. Nasal swab samples were processed for virus isolation in 9-10-day old Specific Pathogen Free (SPF) embryonated chicken eggs. Meanwhile, viral RNA was directly extracted from the nasal swabs samples and subjected to RT-PCR. The full-length HA1 gene was PCR amplified with a set of primers (forward: 5'-CAGAATTCATGGATTCCAACACTGTGT C-3' and reverse: 5'-AACTCGAGTCA TTTCTGCTTTG AAGGGA-3') designed based on the published EIV sequence online (http://www.ncbi.nlm.nih.gov/).

Antigenic analysis: To differentiate the subtype of these two isolates, the collected horse sera were used in HI assays according to standard procedures. Viruses were tested against antiserums specific for American (Miami/ 63, Xinjiang/3/07, Heilongjiang/1/2010, Heilongjiang/ 1/2011) and European (Qinghai/94) representative viruses. Serums of the natural infected horses were obtained by our laboratory when the epidemics generated, including horse serums against Xinjiang/3/07, Heilongjiang /1/2010 and Heilongjiang/1/2011. Besides, serums of guinea pigs against Qinghai/94 and Miami/63 were collected via immunization with corresponding antigens.

Genetic analysis: The HA1 sequences of Heilongjiang/ 1/2010 and Heilongjiang/1/2011 were submitted to GenBank (accession Nos. JQ265982 and JQ265983). To analysis the genetic relationships of the two EIV isolates, the HA1 genes of Heilongjiang/1/10 and Heilongjiang/ 1/11 were compared to other thirty-five sequences, including some EIVs isolated in China and other countries and vaccine isolates recommended by OIE (Newmarket/ 1/93, Newmarket/2/93, Kentucky/1/94, Ohio/ 1/03, Africa/4/03 and Richmond/1/07). At last, an unrooted phylogenetic tree employing neighbor joining (NJ) bootstrap analysis was generated using MEGA 4.0. The statistical validity was supported by the bootstrap values based on 1000 replications.

RESULTS AND DISCUSSION

Two field EIV strains were obtained from two restricted outbreaks in Heilongjiang Province during 2010 and 2012, and were designated as A/equine/ Heilongjiang/1/2010 and A/equine/ Heilongjiang/1/2011, respectively. The two strains were antigenic characterized by HI assay (Table 1). The results of serums of Heilongjiang/1/2010, Heilongjiang/1/2011 against Xinjiang/3/07(256, 256) and Qinghai/94(64, 64) indicated both viruses were of American rather than European lineage. Meanwhile, HI titers of Heilongjiang/1/2010, Heilongjiang/1/2011 against serum of Xinjiang/ 3/07 (512,512), Qinghai/94(64,32) and Miami/63(32,16) suggested antigenic difference existed between them and field EIVs in China evolve constantly under host immunity pressure. Also, antigenic analysis revealed no much antigenic difference occurred between Heilongjiang /1/2010 and Heilongjiang/1/2011, but when subjected to Xinjiang/3/07, some antigenic difference appeared.

Table 1: The results of HI titers using EIV isolates and three reference viruses against corresponding serums

Viruses	Serums				
	Heilongjiang/ 1/2011	Heilongjiang/ 1/2010	Xinjiang/ 3/07	Qinghai/ 94	Miami/ 63
Heilongjiang/1/2011	512	512	512	32	16
Heilongjiang/1/2010	512	512	512	64	32
Xinjiang/3/07	256	256	1024	256	32
Qinghai/94	64	64	256	4096	32
Miami/63	32	32	64	128	2048

The results of virus against homologous serums are shaded; two new isolates are indicated in **bold.**

To perform phylogenetic analysis of HA1 gene of A/equine/Heilongjiang/2010 and A/equine/ Heilongjiang/ 2011, a neighbor-joining phylogenetic tree was constructed (Fig. 1). Different from Tokyo/3/08 responsible for the 2008 EIV epidemic in Japan belonging to Florida sublineage clade 1, the two newly emerging strains clustered in a same branch of Florida sublineage clade 2 with the causative strains of 07-08 EIV outbreak in China, such as Heilongjiang/10/08, Xinjiang/1/07, Hubei/6/08, Gansu/7/08, Huabei/1/07, Inner Mongolia/ 8/08 and Mongolia1/08. Richmond/1/07 was the only one located in Florida sublineage clade 2 among vaccine strains listed by OIE.

Comparisons of amino acid sequences of two EIV isolates with other H3N8 EIVs revealed both viruses were of Florida 2 sublineage of American lineage, showing more than 99% homology with Chinese isolates during 2007-2008 (data not shown). Aligned with conserved HA1 gene, no deletion or insertion of amino acid substitutions were found in Heilongjiang/1/10 and Heilongjiang/1/11. Previous study determined five deduced antigenic sites on the HA protein, named sites A, B, C, D and E, all of which were located in HA1 gene (Damiani *et al.*, 2008). The two Chinese strains, Heilongjiang/1/2010 and Heilongjiang/1/2011, differed by four AA (S47T, T144A, G158E and G198E), and three of which, position 144,158 and 188 were located in deduced antigenic sites. Unique amino acid change was found in HA1 gene of Heilongjiang/1/10 (A144T, Site A) and Heilongjiang/1/11 (G158E, Site B).Meanwhile, unique change (E198G, Site B) was found only in Heilongjiang/1/11 and Heilongjiang/10/08. Amino acid substitution at position 47 (S→T), located outside of the five antigenic sites, was regarded as a special genetic characteristic of isolates in China (Qi *et al.*, 2010), which was observed in Heilongjiang/1/10 again.

As described in previous research, new drift variants subjected to outbreak of influenza had common characteristics of four or more than four amino acid changes located in two or more than two of the five antigenic sites (Daly *et al.*, 2011). The results of alignment between new drift variants with vaccine reference strains were revealed in Table 2. It can be concluded that vaccine containing Richmond/1/07-like antigen has better protective effect in China. But such EI vaccine has not appeared.

The most significant cause for 2008 outbreak of China is likely to be limited EI vaccination programs. Up

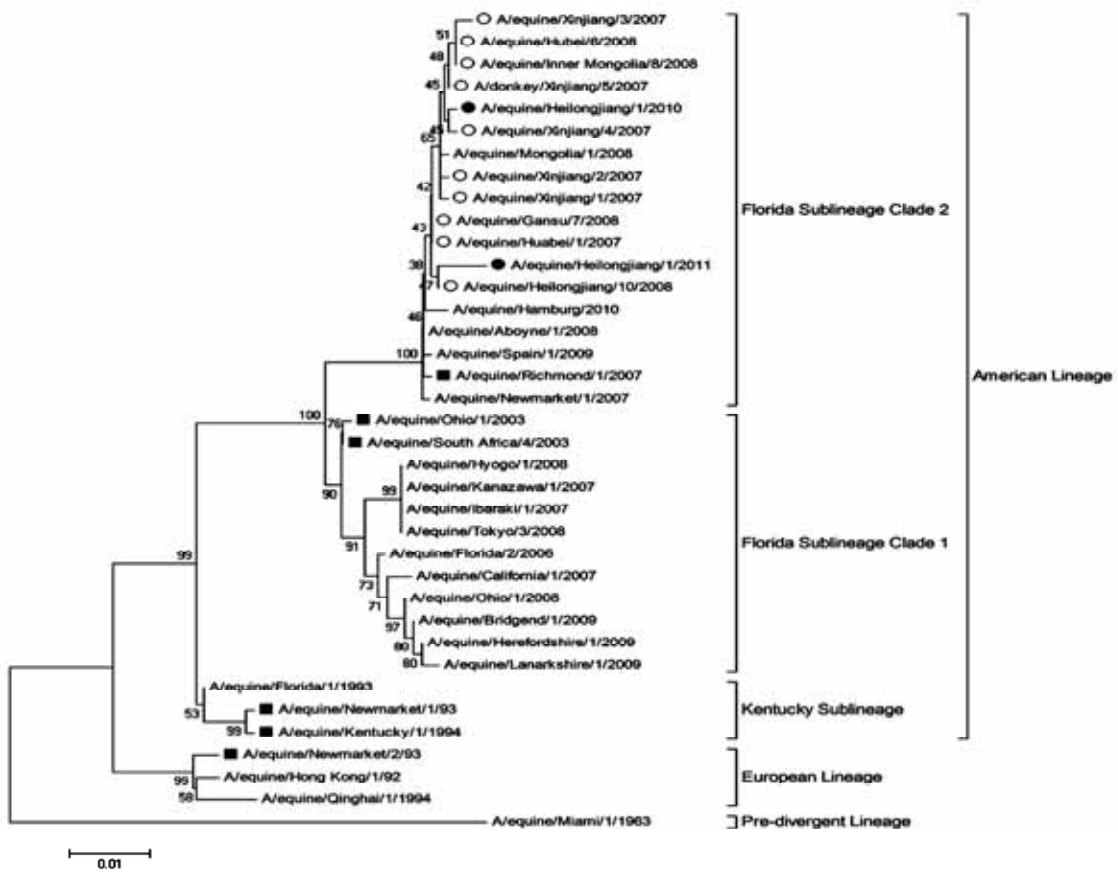

Fig. 1: Based on maximum composite likelihood model, a neighbor-joining phylogenetic tree is constructed by HA1 subunit proteins of selected thirty-five H3N8 EIVs. The two new isolates are shown in a *fixed circle*. The strains isolated from 2007-2008 outbreaks in China are indicated by an *open circle*. Vaccine isolates recommended by OIE are indicated by a *square*.

Table 2: The comparison result of Heilongjiang/1/10 and Heilongjiang/ 1/11 with vaccine reference isolates in five deduced antigenic sites in HA1 subunit protein.

Vaccine reference isolate	New isolate											
	Heilongjiang/1/2010						Heilongjiang/1/2011					
	A	B	C	D	E	Total*	A	B	C	D	E	Total*
Newmarket/2/93	1	2	3	2	1	9/5	2	2	3	2	1	10/5
Newmarket/1/93	-	3	1	-	-	4/2	1	3	1	-	-	5/3
Kentucky/1/94	-	3	1	-	-	4/2	1	3	1	-	-	5/3
Ohio/1/03	-	2	-	-	1	3/2	1	2	-	-	1	4/3
South Africa/4/03	-	2	-	-	1	3/2	1	3	-	-	1	5/3
Richmond/1/07	-	1	-	-	-	1/1	1	1	-	-	-	2/2

*Total indicates: The number of amino acid residue substitutions in five deduced antigenic sites/the number of changed deduced antigenic sites.

to date, few horses in China are vaccinated with commercially available EI vaccine of none-updated recombinant viral vector vaccine ProteqFlu, most of which are racehorses. Its components are Kentucky/94 and Newmarket/2/93(Bryant *et al*., 2010; Gildea *et al*., 2010a). Different from none-updated ProteqFlu, updated ProteqFlu contains antigen of the Ohio/03 vaccine strain, which has the most closely genetically relationship with recent EIV isolates in China among the commercial vaccines. Therefore, compared with ProteqFlu, it is possible that horses vaccinated with updated ProteqFlu in China can achieve a better protective effect.

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