

Evolution of the Complete Matrix and Matrix1, Matrix2 Gene of H3N8 Equine Influenza Virus

Jie Chen¹ and Xinggang Guo^{2*}

¹The 3rd Hospital Affiliated to Harbin Medical University, Harbin - 1500 01, PR China.

²Surgical Department of Tumor, The 4th Hospital Affiliated to Harbin Medical University, Harbin - 150 001, PR China.

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In our study, the M, M1 and M2 gene of H3N8 equine influenza virus (EIV) were phylogenetic analyzed. Also, the aa sequences of the M1 and M2 proteins were aligned and mapped. The result showed that the M, M1 and M2 gene of all the recent EIV strains of Florida-2 clade isolated after 2007 was under divergent evolution. It evolved into a different clade named Aisan clade, which was distinguished from EIV strains of Florida-2 clade isolated before 2007 and was more genetic similar to the European clade. This was also revealed by aa sequence analysis of M1 and M2 protein. In addition, the phylogenetic tree of M2 gene indicated that it evolved in different pattern from the complete M and M1 gene. The M1 and M2 gene of two horse-derived influenza viruses of A/Swine/Chibi/01/2005 and A/Swine/Anhui/01/2006 was confirmed under frozen evolution here. Our studies enrich our knowledge of the M, M1 and M2 gene of H3N8 EIV and will make foundation of further research on it.

Key words : Matrix Gene, Equine Influenza Virus, Evolution, Phylogenetic Analysis.

Equine influenza (EI) is an acute and highly contagious disease in the horse species. Clinical signs of pyrexia, dyspnoea, coughing, myalgia and anorexia may be observed in the sick horses. EI is one of the horse diseases listed by the World Organisation for Animal Health (OIE) and its caused pathogen is equine influenza virus (EIV), which has been classified as two subtypes of H7N7 and H3N8. The H7N7 EIV has not been isolated after 1979(Webster, 1993), meanwhile the H3N8 always evolve and are circulating worldwide (Bryant *et al.*, 2009; Elton and Bryant; Qi *et al.*). The H3N8 subtype viruses evolve as a single lineage at first and later as two antigenic and genetic different clades: American and European (Bryant *et al.*, 2009; Elton and Bryant; Qi *et al.*).

No divergence of the European clade has been observed and no Eurasian viruses have been isolated after 2007. Meanwhile, the American clade evolves into Kentucky and Florida clades. And the Florida clade further evolves into Florida-1 and -2 clades 2010(Bryant *et al.*, 2009; Elton and Bryant; Qi *et al.*), both of which are responsible for the recent EI outbreaks worldwide.

EIV belongs to the family Orthomyxoviridae, genus Influenza virus A, species Influenza A virus, which has a genome of negative single-stranded RNA with eight gene segments, namely Polymerase B2 (PB2), Polymerase B1 (PB1), Polymerase A protein (PA), Haemagglutinin (HA), Nucleocapsid (NP), Neuraminidase (NA), Matrix protein (M) and Non-structural gene (NS). The M gene codes two different functional proteins derived by splicing of mRNA: M1 and M2. M1 protein constructs the matrix of the virus particle, lying beneath the viral envelope in the form of dimmers and interacting with viral ribonucleoprotein (vRNP). M1 is most abundant protein in virus

* To whom all correspondence should be addressed.
Tel.: +85939051; Fax: +86 85939617;
E-mail: guoxinggang@yahoo.com

particle and plays critical roles in many aspects of virus replication (Noton *et al.*, 2007). M2 protein acts as an ion channel pump to lower or maintain the pH of the endosome. Influenza virus-like particles containing M2 have the ability to induce broadly cross protective immunity (Neirynck *et al.*, 1999).

MATERIALS AND METHODS

The M gene sequences of 50 EIV strains were obtained from the online influenza virus database (<http://www.ncbi.nlm.nih.gov/genomes/FLU/>). In addition, three other influenza viruses derived from EIV were also included in our study: A/Swine/Chibi/01/2005 (China), A/Swine/Anhui/01/2006 (China) and A/canine/Florida/242/2003 (America). Sequence data were compiled and edited using DNASTAR (Madison, WI, USA). Then the data were aligned using Bioedit (version: 7.0.9.0) by Clustal W method. The nucleotide (nt) sequences data only for the coding region were used here, including the complete M gene (982bp) and also the M1 (759bp), M2 genes (294bp). Based on model of maximum composite likelihood, an unrooted phylogenetic tree employing neighbor joining (NJ) bootstrap analysis was generated using MEGA 4.0. The statistical validity of this analysis was supported by the bootstrap values based on 1,000 replications.

RESULTS AND DISCUSSION

As can be seen from phylogenetic tree (Fig.1), the evolution of the complete M and the M1 gene was similar to the EIV evolution pattern

described previously (Elton and Bryant, 2011; Qi *et al.* 2010). However, the complete M and M1 gene of Florida-2 EIV strains after 2007, especially the strains from Asian countries, was located in a single clade and more genetic near to the European clade. Accordingly, this clade was named Asian clade. When subjected to the M2 gene, more interesting phenomena were observed. Different from the M and the M1 gene, the M2 gene evolved from the pre-divergence clade into Florida-1 clade and Florida-2 clade. The Florida-2 clade further evolved into Kentucky clade and another clade, containing strains of Asian and European clade. And, compared with other clades, the M2 gene of the Asian clade strains was more similar to the European clade. However, it was noted that, except the M gene, all the other segments of the Asian clade strains belonged to the Florida-2 clade (the detailed data was not shown here). The genetic analysis above supported the divergent evolution of the M, M1 and M2 gene in the recent EIV of Florida-2 clade. In the phylogenetic analysis, the horse-derived swine and canine influenza was grouped as European and Florida-1 clade, respectively. The only known H3N8 avian-derived EIV strain, A/equine/Jilin/1/1989, was clustered by itself and its M2 gene was more genetic close to the other EIV strains, compared the M and M1 gene.

Based on the analysis above, A/equine/Xinjiang/1/2007, a Chinese field strain isolated during the 2007-2008 outbreak (Qi *et al.*, 2010), was compared with other 49 EIV strains to study the homology of the Asian clade with other clades at the nucleotide (nt) and amino acid (aa) level

Table 1. The homology of nt or aa sequences of the M, M1, M2 gene of A/equine/Xinjiang/1/2007, compared with virus of different clades or the avian-derived EIV, A/equine/Jilin/1/1989

Clade or Virus	The Homology (%)				
	M	M1		M2	
	nt	nt	aa	nt	aa
Aisan clade	99.1-100	99.1-100	99.2-99.6	98.6-100	97.9-100
European clade	97.7-98.2	97.8-98.3	96.8-98.4	96.6-97.6	93.8-95.8
Kentucky clade	97.5-97.7	97.2-97.5	98-98.4	96.6	95.8
Florida-2 clade	97.7	97.6	98.4	96.5	95.8
Florida-1 clade	96.7-97.7	96.7-97.5	97.2-98	96.2-96.4	93.8-95.8
Pre-divergence clade	96.1	96.2	97.2-97.6	95.9	93.8
A/equine/Jilin/1/1989	89.8	88.5	96.4	93.8	91.7

(Table 1). It was found that except A/equine/Jilin/1/1989, the M, M1 and M2 of A/equine/Xinjiang/1/2007 shared more than 95% homology with other EIV strains both at the aa and nt level. When just considering the nt sequence, A/equine/Xinjiang/1/2007 was more genetic similar to the European clade in the three genes. It was in accord with the result of the phylogenetic tree constructed based on the nt sequence. However clades with high homology at the nt level might have relatively low or same homology at the aa level, such as the aa sequence of M2 protein of Kentucky and Florida-2 clade. This suggested difference of synonymous codon usage existed in these clades.

To further study the aa characteristics, all the M1 or M2 sequences were compared with that of A/equine/Miami/1/1963, the first H3N8 EIV isolate (Fig.2, Fig.3). It was found that in the M1 protein, less aa substitutions were located in the sites 100-200, indicating this region was more conserved in the evolution of M1. The Asian clade was clearly distinguished from other clades, in the sites of 11, 80, 95 of M1 and 85, 89 of M2. Meanwhile, the European clade could be distinguished from other clades in the site 61 of M2. Some unique aa changes were also observed in some clades, such as, aa substitution of K18N, F48S and L59M in the Asian clade of M2. It was an

interesting phenomenon that in the M2 aa sequence, the aa sequence of strains belonging to the Kentucky clade was completely same as Florida-2 clade, though their nt sequences were different. The HA protein of strains of A/Swine/Chibi/01/2005 A/Swine/Anhui/01/2006 was identified under frozen evolution in previous studies, both of which were genetic similar to A/equine/Qinghai/1/1994(Qi *et al.*). It was identified here the M1 and M2 also experienced the process of frozen evolution. As shown in the Fig. 2, unique aa changes of A143S, T218I, G228R, A239V in M1 and P10S, E70D in M2 were found in the three strains.

The complete M gene of EIV has been genetic and phylogenetic characterized in 1998(Lindstrom *et al.*, 1998). It was reported that before 1998, the M gene of the American clade evolved as a single lineage (Lindstrom *et al.*, 1998). Here, the M gene was classified into the Kentucky, Florida-1 and -2, and Asian clades, indicating the uneven evolution of M gene. Meanwhile, it was noted that all the complete M gene (982bp) of EIV isolates after 2007 available online was included in our analysis. And the M gene of all the circulating strains was finally grouped as two clades: Florida-1 and Asian clades, whose the other genes were of Florida-1 and -2 clades respectively. It was hard to

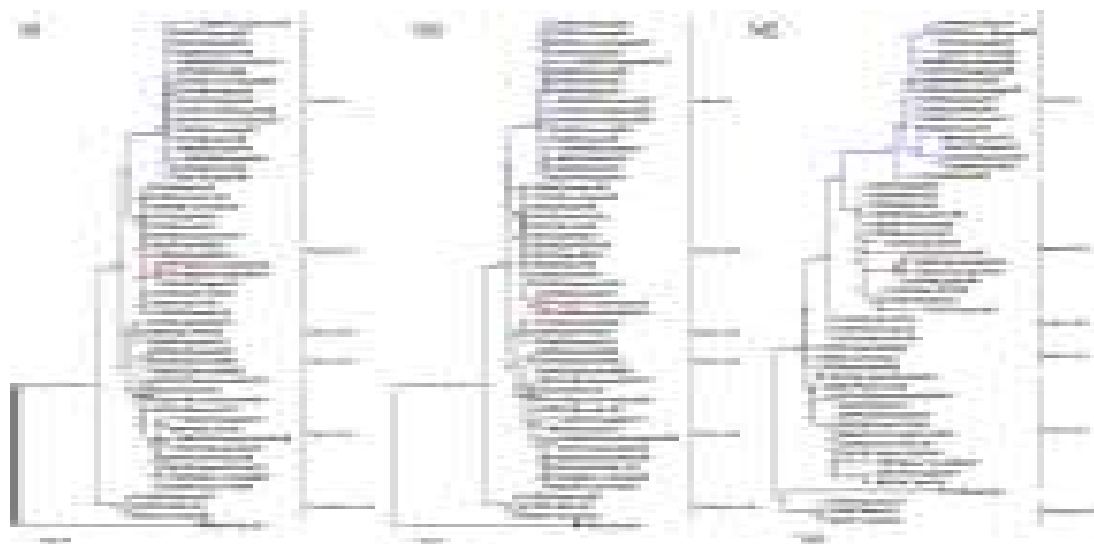


Fig. 1. Based on maximum composite likelihood model, a neighbor-joining phylogenetic tree constructed by the nt sequences of the M, M1, M2 gene of H3N8 EIVs or horse-derived influenza virus.

Bootstrap values are obtained from 1000 replicates. The swine and canine influenza viruses are shown in a red and green fixed circle, respectively. The avian-derived EIV isolate is indicated by a black fixed circle. The branch line of the Asian clade is marked by blue.



Fig. 2. The result of M1aa residues comparison of A/equine/Miami/1/1963 with other isolates, including viruses of Asian clade (*red*), European clade (*blue*), Kentucky clade (*light green*), Florida-2 clade (*light red*), Florida-1 clade (*green*), Pre-divergence clade (*brown*)

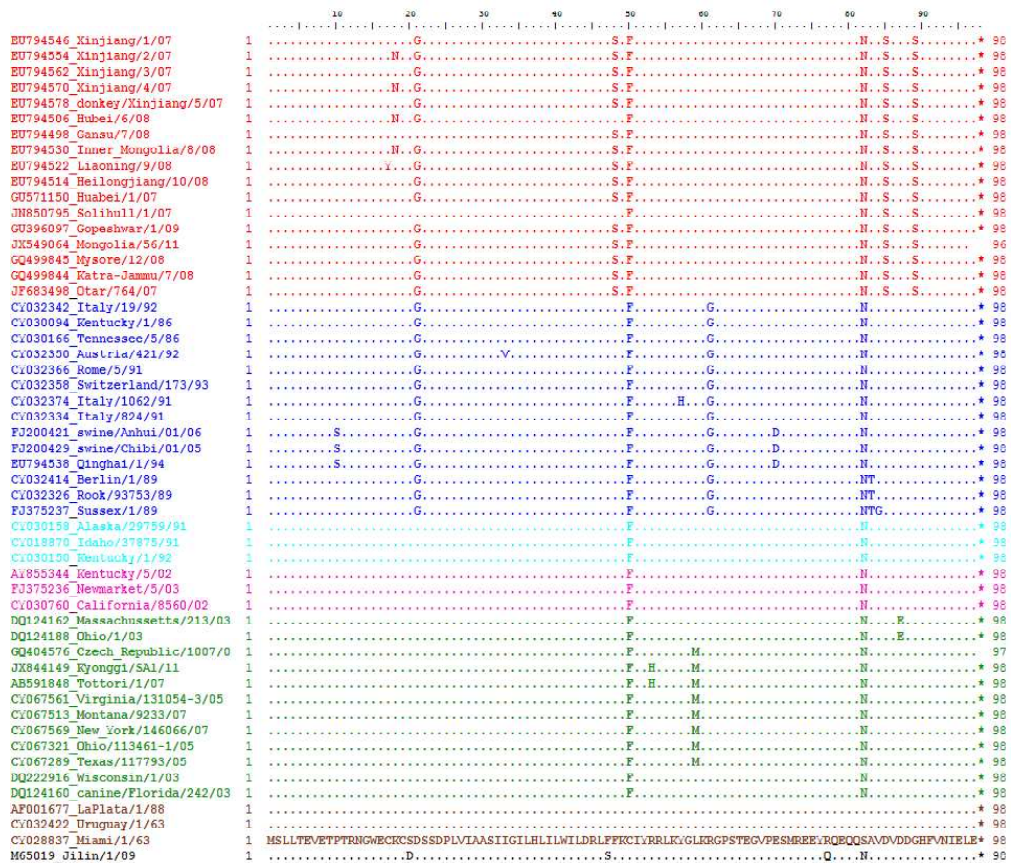


Fig. 3. The result of M2 aa residues comparison of A/equine/Miami/1/1963 with other isolates

interpret the divergent evolution. However, the divergent evolution happened in 2007 or before 2007, as can be indicated in Fig. 1. The Asian clade was genetic similar to the European clade and the last European clade EIV was isolated in 2007. Possibly, the M gene of the Asian clade was obtained from the circulating European clade at the same time and then spread in the horse population. In addition, though most strains of Asian clade were isolated in the Asian countries, one strain of A/equine/solihull/1/2007, was isolated in England (Bryant *et al.*, 2009). This suggested that EIV of the Asian clade might circulate in the European countries.

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