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Genetic evolution of equine influenza virus strains (H3N8) isolated in France from 1967 to 2015 and the implications of several potential pathogenic factors

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ABSTRACT

Equine influenza virus (EIV) is a major respiratory pathogen of horses despite the availability of equine influenza vaccines. This study aimed to determine genetic evolution of EIV strains in France between 1967 to present. A whole genome comparative analysis was also conducted on recent French strains in order to identify potential factors of pathogenicity. Comparison of French EIV sequences with vaccine and worldwide epidemic strains revealed amino acid substitutions in both haemagglutinin (HA) and neuraminidase, especially within the antigenic sites and/or close to receptor binding sites (HA). Amino acid substitutions were also identified in other genes, mainly the polymerase complex proteins and PB1-F2. Viruses belonging to Eurasian and American lineages have circulated until 2003 and Florida sub-lineage Clade 2 strains predominates since 2005. The last French strain (2015) displayed several specificities in HA suggesting the occurrence of antigenic drift with presence of pathogenic markers in the PA and PB1-F2 genes.

1. Introduction

Equine influenza virus (EIV) is an influenza A virus belonging to the *Orthomyxoviridae* family and is considered to be one of the respiratory pathogens causing the greatest economic losses to the equine industry. Equine influenza virus is highly contagious, endemic in most parts of the world and has the potential to disrupt major equestrian events (Landolt et al., 2013). H3N8 EIV was first isolated during an outbreak in Miami (USA) in 1963 and since the late 1980's, has been the only subtype causing major equine outbreaks worldwide (Webster, 1993; Gildea et al., 2011; Legrand et al., 2013; Woodward et al., 2014). The H3N8 subtype has continually evolved, diverging at the end of the 1980's into two antigenically distinct lineages, American and Eurasian. The American lineage has diverged further into Argentinian, Kentucky and Florida sub-lineages (Daly et al., 1996; Lai et al., 2001). Evolution of the Florida sub-lineage has resulted in the emergence of two clades, 1 and 2, which are currently circulating worldwide (Bryant et al., 2009; Gildea et al., 2011; Laabassi et al., 2015; Virmani et al., 2010; Yamanaka et al., 2008). The EIV strains isolated in France typically follow the same evolutionary pattern, but available information is limited (Legrand et al., 2013; Manuguerra et al., 2000).

It is well established that mammalian influenza viruses undergo antigenic drift through amino acid substitutions within the viral glycoproteins haemagglutinin (HA) and neuraminidase (NA). Modifications in the domains carrying one or several antigenic sites can diminish influenza vaccine efficacy and lead to recurrent outbreaks. The HA1 domain appears to be under the most selective pressure induced by the neutralising antibodies (Nelson and Holmes, 2007). The

Abbreviations: EI, equine influenza; EIV, equine influenza virus; HA, haemagglutinin; NA, neuraminidase; PA, polymerase acid; PB1, polymerase basic 1; PB2, polymerase basic 2; M, matrix protein; NS1, nonstructural protein; NEP, nuclear export protein; NP, nucleoprotein; FC1, Florida clade 1; FC2, Florida clade 2

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OIE Expert Surveillance Panel (ESP) annually reviews EIV evolution, especially antigenic drift, in order to recommend updates of the EI vaccine strain composition (OIE World Organisation for Animal Health, 2015) to prevent and mitigate the risks of vaccine breakdown (Woodward et al., 2015). This illustrates the importance of surveillance programs focusing on HA and NA genes encoding two EIV surface glycoproteins. However, other genes are also associated with influenza virus virulence and monitoring of potential novel markers for pathogenicity, such as PB1-F2, may additionally be required Alymova et al. (2014a), (2014b).

This study aimed to compare HA and NA sequences from EIV strains isolated during major French EI outbreaks since 1967 in order to determine their genetic evolution during the last 5 decades. Whole genomes from a panel of recent French EIV strains were also sequenced and potential factors of pathogenicity were highlighted.

2. Materials and methods

2.1. Sample collection

Equine influenza viruses were isolated from sick horses during respiratory disease outbreaks that occurred in France from 1967 to 2003 by the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) and since 2005 by the RESPE-associated veterinarians (Legrand et al., 2013) and LABÉO Frank Duncombe. After collection, nasopharyngeal swabs were placed immediately in 5 ml of virus transport medium containing minimum essential medium supplemented with 10% of fetal bovine serum and 1% antibiotics (Penicillin, Streptomycin and Amphotericin).

2.2. Extraction of nucleic acids and EIV diagnosis

RNA was extracted from 140 µl of respiratory fluids using the QIAamp Viral RNA Mini Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's recommendations. For detection of EIV, two one-step RT-PCR were performed using equine-specific M1 primers: period 2005–2009 (Legrand et al., 2013); period 2010–2016 adapted from (Heine et al., 2007).

2.3. Genes sequencing and analysis

The HA and NA sequences from French EIV outbreak isolates were determined (30 and 28, respectively). The phylogenetic analysis was carried out for HA1 only due to a limited number of complete HA sequences available in GenBank for reference and comparison. The strains A/equine/Jouars/4/2006, A/equine/Belfond/6-2/2009, A/equine/Neuville-Pres-Sees/1/2011, A/equine/Cambremer/1/2012, A/equine/Gironde/1/2014 and A/equine/Saone-et-Loire/1/2015 were fully sequenced.

For EIV strains isolated between 1967 and 2003, viral genome extracted were reverse transcribed into cDNA using SuperScript III (Thermo scientific), cDNA syntheses were performed using the influenza universal primer AGCAAAAGCAGG. PCR amplification from 2.5 µl of cDNA products was performed using the PCR extender system (5Prime) following the manufacturer's instructions. During the first cycle, samples were incubated at 94 °C for 2 min followed by 35 cycles with the following conditions: 94 °C for 20 s, 55 °C for 20 s, 72 °C for 2 min. The program ended with one cycle at 72 °C for 10 min. Primers used for amplification were Eq/H3/9F (CAGGGGATATTT CTGTCAATCATG) or H3HA11F (AGCAAAAGCAGGGGATATTTCTG) (Ilobi et al., 1998) and Eq-H3-1741R (AGTAGAAACAAGGGT GTTTTTAAC) or EH3-1686R (GCATCTGATGTTGCCTTTTTGGC) for HA gene and Eq/N8/2F (GCAAAAGCAGGAGTTTAAAATG) and Eq/N8/1460R (GTAGAAACAAGGAGTTTTTTTCG) for NA gene (Manuguerra et al., 2000).

For EIV strains isolated since 2005, genes were amplified in two-steps after extraction. The first step was the reverse transcriptase using Superscript II (Thermo Scientific) followed by an amplification (second step). PCR products were generated using Phusion Hot Start II high-fidelity DNA polymerase (Thermo Scientific). HA and NA were amplified according to Cullinane process (Cullinane, personal communication) and other genes (PB2, PB1, PA, NP, M and NS) with M13 primer sequences as described previously (Rash et al., 2014). Sequencing was performed by Biofidal (Vaulx en Velin, France) using the Sanger method. Sequences were assembled and contigs were analysed with the CodonCode Aligner v1.5.2 software (CodonCode Corporation, Dedham, USA). A multiple alignment of all sequences was conducted using the Muscle algorithm and neighbor-joining trees method (MEGA V6 software (Tamura et al., 2013)), with a maximum likelihood substitution model and bootstrapped 1000 times to assess the reliability. Additional strains were added to compare conserved mutations between the French EIV strains and vaccine or outbreak strains, such as A/equine/Hong Kong/1/1992, A/equine/Sydney/6085/2007 and A/equine/Dubai/1/2012. The amino acid sequences of other viral proteins (PB2, PB1, PB1-F2, PA, PA-X, NP, M1, M2, NS1, NS2-NEP) from recent EIV isolated in France were aligned with the amino acid OIE-ESP recommended strain A/equine/Richmond/1/2007. The strain A/equine/Jouars/4/2006 was not analysed for PB1-F2 due to lack of the beginning of the nucleic acid sequence of the segment PB1. The workflow is summarised in the Supplementary Fig. 1.

3. Results

3.1. Phylogenetic analysis

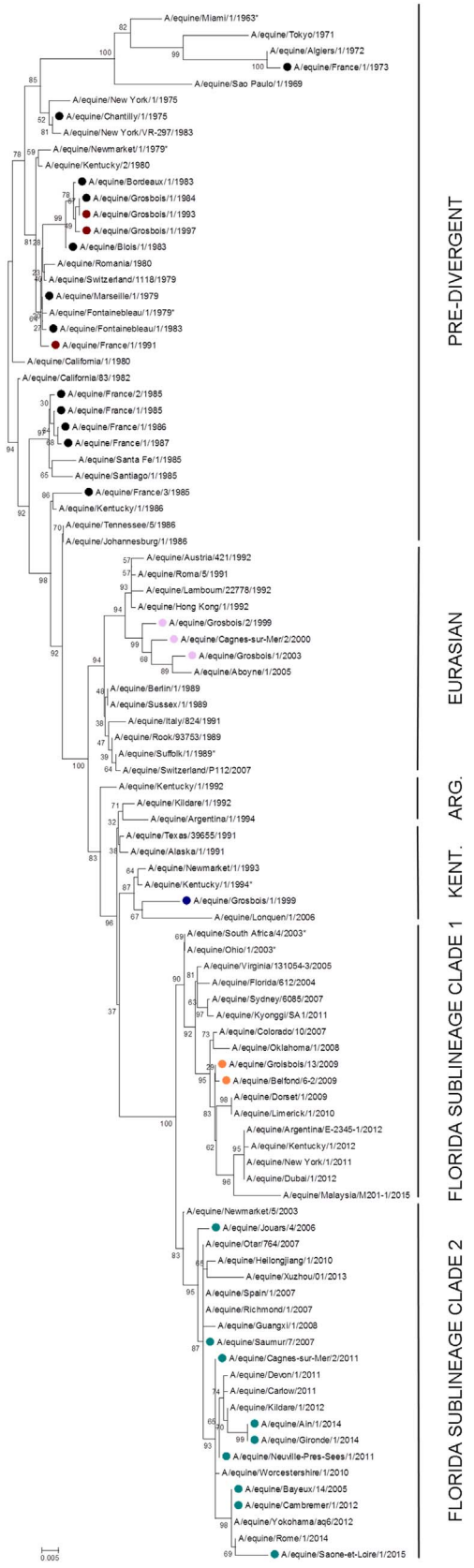
Analysis of the HA1 nucleotide sequences generated from the French EIV strains and other EIVs retrieved from GenBank showed six distinct clusters. The 30 French EIV strains analysed were located within 5 out of the 6 clusters Fig. 1. The HA1 sequences of the 15 viruses isolated between 1973 and 1997 were found within the Pre-divergent lineage. While the 3 viruses isolated between 1999 and 2003 belonged to Eurasian lineage. An EIV strain belonging to the Florida Clade 1 was isolated during the Grosbois epidemic in 2009. The strain A/equine/Grosbois/1/1999 was located within the Kentucky lineage. From 2005, nine EIV isolates analysed in this study belonged to Clade 2 of the Florida sub-lineage. Overall, these observations for the HA1 gene were correlated with the complete NA sequence analysis, illustrated in Fig. 2. However, we observed a lack of homogeneity for the strain A/equine/Cambremer/1/2012 between HA1 and NA sequence analyses (99% for HA1 and 98% NA compared to A/equine/Saone-et-Loire/1/2015).

3.2. Comparison of HA amino acid alignment

The HA amino acid sequences of the 30 French EIV strains isolated since the early 70 s were compared to A/equine/Miami/1/1963. The amino acid substitutions are represented in Table 1 and Supplementary Fig. 2.

In most cases, when a specific amino acid substitution has occurred in several strains of the Pre-divergent lineage, it was conserved into other clusters (i.e. G6S, T46I, V102I, L111I, M121T, S137G, R140K, E158G, T187S, V196I, A198E, V242I, I267V, V309I, S312N, Q324P, K387R, N464G, D489Y and R503K). However, the amino acid substitutions R140K, T187S, V196I and I267V, which appeared in 1985, have not been found in the later EIV strains A/equine/France/1/1991, A/equine/Grosbois/1/1993 and A/equine/Grosbois/1/1997. The amino acid substitutions T48I and D172N specific to the Pre-divergent lineage, which were located in antigenic sites C and D, respectively, further mutated (I48M andN172K) from 1999 and 1989, respectively. Comparison of HA amino acid sequences showed that in most cases, the substitutions that appeared in the Eurasian and/or

American lineages (i.e. Kentucky and Florida sub-lineages) were subsequently conserved in French strains from the same cluster collected afterwards, such as amino acid substitutions N189Q, R261K and T276I for the American lineage. The amino acid at position 189 also underwent an additional change (N189K) that was only found in the Eurasian lineage. Moreover, this lineage is distinguished from the American lineage by 8 additional amino acid substitutions T163I, N189K, K207E, I213V, P273L, E280K, V300I and M462I. Within the American lineage, representative strains of the Florida sub-lineage Clade 1 and Clade 2 differed by 9 amino acids: 4 specific substitutions of the Clade 1 viruses (R62K, V78A, D104N and V223I) and 5 specific substitutions of the Clade 2 viruses (P103L, V112I, S159N, A372T and G379E). The residue at position 7 showed two substitutions (G7D and G7N), which appear to be representative of clades 1 and 2, respectively. The amino acid substitution G7N was found in all strains belonging to the Florida sub-lineage Clade 2 except for A/equine/Jouars/4/2006 and A/equine/Cagnes-sur-Mer/2/2011, which possess the amino acid substitution G7D found mostly within the strains belonging to the Florida sub-lineage Clade 1. Within this clade, the substitutions R62K and V78A were both found in the antigenic site E. The amino acid substitution S159N, specific to the Florida sub-lineage Clade 2, is located in the antigenic site B. The reverted substitution N159S was observed in all strains belonging to the Florida sub-lineage Clade 1.



The HA1 sequence from the French strain A/equine/Saone-et-Loire/1/2015 differed by 8 residues when compared to other strains belonging to the same clade. This strain possessed the amino acid substitutions T131N, A144T, T179V, T192K, Q197R, I282V, R321K and I527V. The substitutions T179V and T192K were also found in the French strains A/equine/Bayeux/14/2005, A/equine/Cambremer/1/2012 and in the Italian strain A/equine/Rome/1/2014, and the substitution I527V was also present in the French strains A/equine/Grosbois/13/2009 and A/equine/Belfond/6-2/2009 and the Australian strain A/equine/Sydney/6085/2007. The residue 144 underwent another substitution (A144V) in the strains A/equine/Ain/1/2014 and A/equine/Gironde/1/2014. The HA amino acid alignment of the strain A/equine/Saone-et-Loire/1/2015 showed the presence of 3 new substitutions within the antigenic sites A (A144T) and B (T192K and Q197R) and 1 new substitution close to the antigenic site A (T131N).

The study highlighted 204 synonymous/silent mutations. We observed that some of these residues involved in a silent mutation that subsequently underwent another mutation with a change of amino acid, such as observed at position 58 (in all strains belonging to both clades of the Florida sub-lineage), position 110 (in Florida sub-lineage Clade 2 strains isolated since 2011), position 261 (in American lineage strains), position 280 (only in Eurasian lineage strains) and position 379 (in Florida sub-lineage Clade 2 strains).

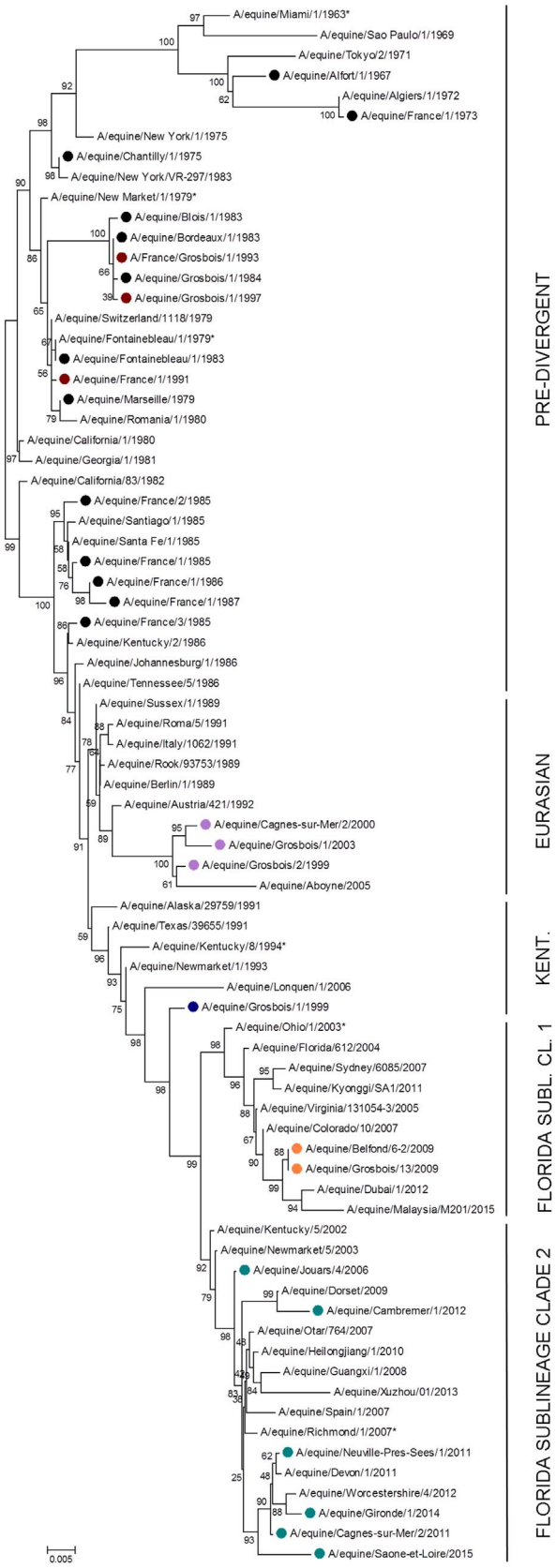
3.3. Comparison of NA amino acid alignment

The NA amino acid sequences of the French strains were compared to A/equine/Miami/1/1963 (Supplementary Fig. 3). Twenty-one amino acid substitutions that appeared within the Pre-divergent lineage were subsequently preserved in other lineages: L17I, V18L, S37N, E76R, S84N, L125S, E160K, V161I, A174S, H205N, V213I, Q250K, N268S, V289I, V301I, S341N, Q342K, N355T, K390R, V394I and V452I. However, residue 17 underwent another amino acid substitution (L17V) which was only observed in the French isolates belonging to the Eurasian lineage. The reverted substitution I452V was observed

Fig. 1. Phylogenetic analysis of the HA1 nucleotide sequence for 95 EIV strains, including representative strains of the main lineages, sub-lineages and vaccine strain (*). The French strains highlighted with a colored dot: French strains belonging to the Pre-Divergent lineage (black) except for the French strains isolated in the 1990s (red), French strains belonging to the Eurasian lineage (purple), French strain belonging to the Kentucky sublineage (blue), French strains belonging to the Florida sublineage clade 1 (orange) and French strains belonging to the Florida sublineage clade 2 (green). Neighbor-Joining, Test phylogeny: Bootstrap method with 1000 bootstrap replication, Mode/method: Maximum composite Likelihood. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0 Tamura K, Stecher G, Peterson D, Filipski A, and Kumar S (2013) Molecular Biology and Evolution 30:2725-2729.

in the strain A/equine/Saone-et-Loire/1/2015. The residue 198 presented a first substitution (A198T) in 1979 followed by a second mutation (T198N) in strains isolated since 1985 except for the French strains isolated in 1991, 1993 and 1997. In regards to the residues at position 125, 161, 174, 205, 268, 289, 342 and 355, the substitutions observed were not present in the four French strains isolated in 1991, 1993, 1997 and 2000. The reverted substitution K342Q and S268N were also found in the French strain A/equine/Grosbois/1/2003 and A/equine/Sydney/6085/2007, respectively. The amino acid substitutions T9A and I70T, appeared within the Pre-divergent lineage but were no longer observed into the Florida sub-lineage Clade 2. This late residue underwent a new substitution (I70A), which was only present within the Florida sub-lineage Clade 2. These substitutions were one of those distinguishing between the current Clade 1 and Clade 2. The amino acid changes representative of current Clade 1 were: S337N (since 2007), S12F, V35A, G40R, G42D, R260K (since 2009), and G416E. This last substitution was also found for the Eurasian strain A/equine/Grosbois/2/1999. The amino acid substitutions G40E, Y66H, S78P, R109K (from 2011 with the exception of A/equine/Cambremer/1/2012), V191I (also observed for 5 strains belonging to the Pre-divergent lineage) and D235N (substitution also found in French strains isolated in 1986 and 1987) seems to be representatives of the Clade 2. Moreover, the strain A/equine/Gironde/1/2014 presents three novel mutations at NA positions 25,42 and 48. The importance of these mutations for NA is unknown.

This comparison of sequences highlighted 164 synonymous/silent mutations subsequently displayed non-synonymous substitutions.



3.4. Comparison of other viral proteins

The amino acid sequences of other viral proteins (PB2, PB1, PB1-F2, PA, PA-X, NP, M1, M2, NS1, NS2-NEP) from recent French EIV strains Clade 2 (the oldest was A/equine/Jouars/4/2006) were compared with the strain A/equine/Richmond/1/2007, an OIE-recommended Florida Clade 2 strain for EI vaccine composition (Fig. 3). The EIV strain A/equine/Saone-et-Loire/1/2015 exhibited more important changes in its protein sequences relative to A/equine/Richmond/1/2007. This strains from 2015 possess amino acid substitutions in the polymerase basic 1 (PB1; 13 substitutions) and the polymerase acid (PA; 11 substitutions). Some substitutions were highlighted for all 5 French EI strains FC2 but only few of them were preserved in the strains isolated since 2011, such as PB2 S107N, PB1 T221A, PB1-F2 D50V, PA K158R, NP N473S, M1T168I and M2 D21G.

The analysis of the gene product PB1-F2, which is encoded by a +1 reading frame shift of PB1, showed a large number of substitutions. PB1-F2 is the protein which presents the highest percentage of substitutions (9.9%) when compared to other proteins (M2=8.3%; M1=3.6%; NS1=3.2%; PA=2.5%; PB1=2.5%; NS2-NEP=1.7%; PA-X=1.6%; NP=1.4% and PB2=1.2%). HA and NA remains however, proteins that have the highest percentage of substitutions (HA=12.2%; NA=11.9%).

The PB1-F2 analysis showed that the sequences of the EIV strains A/equine/Cambremer/1/2012 and A/equine/Saone-et-Loire/1/2015 differ at a terminal position (amino acid residue 75). The amino acid substitution R75H was present only in this two French strains. PB1-F2 from A/equine/Saone-et-Loire/1/2015 is 90 amino acids long when compared with an 81 amino acid length for the other strains (Fig. 4).

Concerning the French strain belonging to Florida sub-lineage

Fig. 2. Phylogenetic analysis of the NA nucleotide sequence for 77 EIV strains, including representative strains of the main lineages, sub-lineages and vaccine strain (*). The French strains highlighted with a colored dot: French strains belonging to the Pre-Divergent lineage (black) except for the French strains isolated in the 1990s (red), French strains belonging to the Eurasian lineage (purple), French strain belonging to the Kentucky sublineage (blue), French strains belonging to the Florida sublineage clade 1(orange) and French strains belonging to the Florida sublineage clade 2 (green). Neighbor-Joining, Test phylogeny: Bootstrap method with 1000 bootstrap replication, Mode/method: Maximum composite Likelihood. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0 Tamura K, Stecher G, Peterson D, Filipski A, and Kumar S (2013) Molecular Biology and Evolution 30:2725-2729.

Table 1
Amino acid alignments of five antigenic sites A to E between HA sequences determined for French strains (30 strains) and vaccine strains (*) or viral strains causing large-scale outbreaks worldwide (°) compared with A/equine/Miami/1/1963. The antigenic sites defined for human H3 influenza virus was used as a reference (Both et al., 1983).

	Site A			Site B		Site C		Site D			Site E	
Human H3 antigenic sites	121-126	132-137	142-146	155-163	187-199	48-55	273-278	170-174	201-218	241-248	62-63	78-83
A/equine/Miami/1/1963	MAEGFT	QNGGSS	GSADS	TKSESSYPT	TNNEQTKLYVQAS	TGKICNNP	PIDTCV	NNDNF	RVTVSTKRSQQTIIPNIG	DVLMINSN	RN	VFQYEN
A/equine/France/1/1973	.....	...R..	.....	.....S.	.....	.....	.....	.....	.....L....	...V....	K.	...H.K
A/equine/Chantilly/1/1975	T.....	...I.G	.....	...GN....	.....E.	I.....	.....	..N..	.....	.....	..	.....
A/equine/Marseille/1/1979	T.....	...R.G	.....	...GN....	.....EL	I.....	.....	..N..	.....	.I.....	..	.....
A/equine/Newmarket/1/1979*	T.....	...R.G	.....	...GD....	.....E.	I.....	.....	..N..	.....	.I.....	..	.....
A/equine/Fontainebleau/1/1979*	T.....	...R.G	.....	...GN....	.....EL	I.....	.....	..N..	.....	.I.....	..	.....
A/equine/Bordeaux/1/1983	T.....	...R.G	.....	...GN....	.....EL	I.....	.....	..N..	.....	.I.....	..	A...K.
A/equine/Fontainebleau/1/1983	T.....	...R.G	.....	...GN....	.....EL	I.....	.....	..N..	.....	.I.....	..	.....
A/equine/Blois/1/1983	T.....	...R.G	.....	...GN....	.....EL	I.....	.....	..N..	.....	.I.....	..	A...K.
A/equine/Grosbois/1/1984	T.....	...R.G	.....	...GN....	.....EL	I.....	.....	..N..	.....	.I.....	..	A...K.
A/equine/France/1/1985	T.....	...R.G	.....	...GN....	S.....I.E.	I.....	.....	..N..	.....	.I.....	..	.....
A/equine/France/2/1985	T.....	...R.G	.....	...GN....	S.....I.E.	I.....	.....	..N..	.....	.I.....	..	.....
A/equine/France/3/1985	T.....	...R.G	.....	...GN....	SS.....I.E.	I.....S	.....	..N..	.....	.I.....	..	.....
A/equine/France/1/1986	T.....	...R.G	.....	...GN....	S.....I.E.	I.....	.....	..N..	.....	.I.....	..	.....
A/equine/France/1/1987	T.....	...R.G	.....	...GN....	S.....I.E.	I.....	.....	..N..	.....	.I.....	..	.....
A/equine/Suffolk/1/1989*	T.....	...R.G	.....	....N...I	S.K.....I.E.	I.....S	.....	..K..	.....E.....V.....	.I.T....	..	.....
A/equine/France/1/1991	T.....	...R.G	.....	...GN....	.....EL	I.....	.....	..N..	.....	.I.....	..	.....
A/equine/Hong Kong/1/92°	T.....	...R.G	.....	...GN...I	S.K.....I.E.	I.....S	L.....	..K..	.....E.....V.....	.I.T....	..	D.....
A/equine/Grosbois/1/1993	T.....	...R.G	.....	...GN....	.....EL	I.....	.....	..N..	.....	.I.....	..	A...K.
A/equine/Newmarket/1/1993°	T.....	...R.G	.....	...GN....	S.QQ..E..I.E.	I.....S	...I..	..K..	.....	.I.....	..	.....
A/equine/Kentucky/1/94*	T.....	...R.G	.....	...GN....	S.QQ..E..I.E.	I.....S	...I..	..K..	.....	.I.....	..	.....
A/equine/Grosbois/1/1997	T.....	...R.G	.....	...GN...I	.....EL	I.....	.....	..N..	.....	.I.....	..	A...K.
A/equine/Grosbois/1/1999	T.....	...R.G	.....	...GN....	S.QQ..E..I.E.	I.....S	...I..	..K..	.....	.I.....D	..	.....
A/equine/Grosbois/2/1999	T.....	...R.G	.....	....N...I	S.K.....I.E.	M.....S	L.....	..K..	.....E.....V.....	.....	..	D....S
A/equine/Cagnes-sur-Mer/2/2000	T.....	.....G	.....	...GN...I	S.K.....I.E.	M.....S	L.....	..K..	.....E.....V.....	.....	..	D.....
A/equine/Grosbois/1/2003	T.....	...R.G	.....	...GN...I	S.....I.E.	M.....S	L.....	..K..	.....	.....	..	D....S
A/equine/South Africa/4/2003*	T.....	...R.G	.....	...G.....	S.Q.....I.E.	M.....S	...I..	..K..	.....E.....V.....	.I.....	..	A.....
A/equine/Ohio/1/2003*	T.....	...R.G	.....	...G.....	S.Q.....I.E.	M.....S	...I..	..K..	.....	.I.....	..	A.....
A/equine/Bayeux/14/2005	T.....	...R.G	.....	...GN....	S.Q..K...I.E.	M.....S	...I..	..K..	.....	.I.....	..	.....
A/equine/Jouars/4/2006	T.....	...R.G	.....	...GN....	S.Q.....I.E.	M.....S	...I..	..K..	.....	.I.....	..	.....
A/equine/Saumur/7/2007	T.....	...R.G	.....	...GN....	S.Q.....I.E.	M.....S	...I..	..K..	.....M.....	.I.....	..	.....
A/equine/Richmond/1/2007*	T.....	...R.G	.....	...GN....	S.Q.....I.E.	M.....S	...I..	..K..	.....	.I.....	..	.....
A/equine/Sydney/6085/2007°	T.....	...K.G	.....	...G...S.	S.K.....I.E.	M.....S	...I..	..K..	.....	.I.....	..	A.....
A/equine/Grosbois/13/2009	T.....	...R.G	.....	...G.....	S.Q.....I.E.	M.....S	...I..	..K..	.....	.I.....	K.	A.....
A/equine/Belfond/6-2/2009	T.....	...R.G	.....	...G.....	S.Q.....I.E.	M.....S	...I..	..K..	.....	.I.....	K.	A.....
A/equine/Cagnes-sur-Mer/2/2011	T.....	...R.G	.....	...GN....	S.Q.....I.E.	M.....S	...I..	..K..	.....M.....	.I.....	..	.....
A/equine/Neuville-Près-Sées/1/2011	T.....	...R.G	.....	...GN....	S.Q.....I.E.	M.....S	...I..	..K..	.....	.I.....	..	.....
A/equine/Cambremer/1/2012	T.....	...X.G	.....	...GN....	S.K.....I.E.	M.....S	...I..	..K..	.....	.I.....	..	.....
A/equine/Argentina/E-2345-1/2012°	T.....	...R.G	.....	...G.....	S.Q.....I.E.	M.....S	...I..	..K..	.....	.I.....	K.	A.....
A/equine/Dubai/1/2012°	T.....	...R.G	.....	...G.....	S.Q.....I.E.	M.....S	...I..	..K..	.....	.I.....	K.	A.....
A/equine/Ain/1/2014	T.....	...R.G	..V..	...GN....	S.Q.....I.E.	M.....S	...I..	..K..	.....	.I.....	..	.....
A/equine/Gironde/1/2014	T.....	...R.G	..V..	...GN....	S.Q.....I.E.	M.....S	...I..	..K..	.....	.I.....	..	.....
A/equine/Rome/1/2014	T.....	...R.G	.....	...GN....	S.K.....I.E.	M.....S	...I..	..K..	.....	.I.....	..	.....
A/equine/Saône-et-Loire/1/2015	T.....	...K.G	..T..	...GN....	S.Q..K...IRE.	M.....S	...I..	..K..	.....	.I.....	..	.....
A/equine/Malaysia/M201-1/2015	T.....	...R.G	.....	...G.....	STQ.....I.E.	M.....S	...I..	..K..	.....	.I.....	K.	A.....

Clade 1A/equine/Belfond/6-2/2009, this strain possessed a low number of substitutions compared to A/equine/Ohio/1/2003, an OIE-recommended Florida Clade 1 strain for EI vaccine composition (Fig. 5). Only PB2, PB1, PA, PA-X, PB1-F2, M2 and NS1 proteins are concerned. Like for the French Florida Clade 2 strains (with the exception of A/equine/Saone-et-Loire/1/2015), PB1-F2 from A/equine/Belfond/6-2/2009 possessed a sequence of 81 amino acids (Fig. 4).

4. Discussion

Since the first isolation of EIV subtype H3N8 in Miami 1963, several major outbreaks have occurred, most notably, Hong Kong 1992 (Watkins et al., 1993), Sydney 2007 (Watson et al., 2011) and Dubai 2012 (Woodward et al., 2014). Despite the development and commercialisation of EI vaccines, EIV continues to circulate in many countries, suggesting that vaccine coverage is sub-optimal and/or the viruses undergo significant antigenic drift.

The EIV outbreak strains that were isolated in France for the past 5 decades illustrate the evolution of H3N8 viruses. Results are consistent with previous studies (Damiani et al., 2008; Murcia et al., 2011) that have determined lineages distinguished by both decade and location. Thereby, the phylogenic analysis demonstrated that strains isolated between 1967 and 1997 belong to Pre-divergent lineage whereas strains responsible for the outbreaks between 1999 and 2003 belong to the Eurasian lineage. However, the first appearance of EIV strains belonging to the Eurasian lineage appeared to be delayed in France when compared to the rest of Europe. It is surprising to observe that the strains isolated from 1991 to 1997 in France were still present within the Pre-divergent lineage, which is closer to strains isolated in the early 80 s, while other strains isolated in Europe at this period belonged to the Eurasian lineage (Borchers et al., 2005; Lewis et al., 2011). Early amino acid substitutions within the French strains were consistent with recently published observation (Woodward et al., 2015). In our study, we found the same amino acid substitutions for the HA at position 46, 55, 140, 159, 163, 172, 187, 189, 196, 207, 213, 260, 267 and 310. These amino acid changes appeared in most of the cases during the mid 80's and were subsequently conserved within the Eurasian and American lineages except for the amino acid substitutions T163I, K207E and I213V, present only in the strains belonging to the Eurasian lineage. The P55S, substitution which appeared in 1985, is very interesting as it causes the occurrence of a new glycosylation site and could play a major role by masking the antigenic site C to antibody recognition (Munk et al., 1992; Skehel et al., 1984).

In 1999, EIV strains from both the Eurasian and Kentucky lineages co-circulated in France. This was the first isolation of a strain belonging to an American lineage in France. It is interesting to note that the majority of substitutions which occurred within the Pre-divergent Eurasian and Kentucky lineages were either conserved in time or replaced by a new amino acid substitution as it was the case for HA residues at position 48, 78, 172 and 189. This last amino acid is involved in the receptor binding site for sialic acid (including the HA 190-helix) (Koel et al., 2013) and is often responsible for major antigenic change in association with others substitutions (such as

Gene	PB2									PB1																			
Amino acid position	9	22	107	122	152	218	377	674	686	1	63	114	168	183	216	221	329	377	430	431	436	437	442	443	445	446	464	736
A/equine/Richmond/1/2007	D	K	S	V	A	L	A	A	V	M	A	I	K	T	S	T	R	E	K	Y	Y	W	Q	S	D	D	D	K
A/equine/Jouars/4/2006	D	K	S	I	A	L	T	A	V						S	T	R	D	K	Y	Y	W	Q	S	D	D	D	R
A/equine/Neuville-Pres-Sees/1/2011	D	R	N	V	A	L	A	A	V	M	A	I	R	T	S	A	R	E	K	Y	Y	W	Q	S	D	D	D	K
A/equine/Cambremer/1/2012	D	K	N	V	A	L	A	A	V	M	A	I	K	T	S	A	R	E	K	Y	Y	W	Q	S	D	D	D	K
A/equine/Gironde/1/2014	N	R	N	V	V	V	A	A	V	K	V	I	R	A	S	A	R	E	K	Y	Y	W	Q	S	D	D	D	K
A/equine/Saone-et-Loire/1/2015	D	K	N	V	A	L	A	V	I	M	A	V	K	T	C	A	Q	E	T	N	G	R	H	R	V	Y	N	K

Gene	PA																	PA-X				PB1-F2							
Amino acid position	7	27	99	100	158	162	198	199	210	213	224	237	277	353	379	565	588	605	198	199	213	237	7	11	19	21	32	50	69	75
A/equine/Richmond/1/2007	Q	D	R	A	K	T	E	E	M	K	S	K	H	R	V	V	S	K	K	R	S	N	T	L	Q	R	H	D	P	R
A/equine/Jouars/4/2006	Q	D	R	V	K	T	D	D	T	M	S	N	H	R	V	V	S	K	M	T	C	I								
A/equine/Neuville-Pres-Sees/1/2011	Q	E	R	A	R	T	E	E	T	K	S	K	H	K	V	V	S	K	K	R	S	N	T	L	Q	R	H	V	P	R
A/equine/Cambremer/1/2012	Q	N	R	T	R	T	E	E	T	K	S	K	H	R	V	V	S	K	K	R	S	N	T	L	Q	R	H	V	P	H
A/equine/Gironde/1/2014	Q	E	K	A	R	T	E	E	T	K	S	K	H	K	M	V	P	K	K	R	S	N	I	Q	R	R	Y	V	P	R
A/equine/Saone-et-Loire/1/2015	K	N	R	T	R	I	E	E	T	K	P	K	Y	R	V	I	S	R	K	R	S	N	T	L	Q	K	H	V	L	H

Gene	NP							M1									M2								NS1							NEP	
Amino acid position	100	111	117	245	305	396	473	13	14	15	20	37	72	80	95	168	21	54	68	70	85	88	89	92	7	86	90	96	180	197	207	7	14
A/equine/Richmond/1/2007	K	H	M	G	K	T	N	S	I	I	L	T	R	I	K	T	D	R	V	E	S	D	S	V	S	T	L	D	V	T	Y	S	M
A/equine/Jouars/4/2006	K	H	M	G	K	N	N	S	I	V	L	A	Q	V	R	T	D	R	V	E	D	N	G	V	S	T	L	D	V	T	H	S	M
A/equine/Neuville-Pres-Sees/1/2011	K	H	M	G	K	T	S	S	I	I	L	T	R	I	K	I	G	R	I	E	S	D	S	V	S	T	L	D	I	T	H	S	M
A/equine/Cambremer/1/2012	K	H	M	C	K	T	S	S	V	I	L	T	R	I	K	I	G	H	V	K	S				S	T	S	D	V	A	H	S	M
A/equine/Gironde/1/2014	K	Y	M	G	R	T	S	F	I	I	F	T	R	I	K	I	G	R	I	E	S	D	S	V	L	T	L	E	I	T	H	L	T
A/equine/Saone-et-Loire/1/2015	R	H	R	G	K	T	S	F	V	I	L	T	R	I	K	I	G	H	V	K	S	D	S	A	S	I	L	D	V	A	H	S	M

Fig. 3. Amino acid differences between the recent French strains and the OIE-recommended vaccine strain A/equine/Richmond/1/2007.

amino acid at position 158 and 159). This study also showed that the loss of an amino acid substitution by restoring the original residue is a rare phenomenon and was only observed for the residue 159 in all strains belonging to the Florida sub-lineage Clade 1. The amino acid substitution S159N appeared after 1973 and was not found in any strains belonging to the Florida Clade 1. This residue is located within the HA 150-loop described as being involved in the binding site of neutralising monoclonal antibodies (Lee et al., 2012). The sequences and conformations of the receptor binding site outer loops are essential for binding and the main antibody target. A change in the sequence can lead to immune escape. It appears that the amino acid Gly158 interacts with heterosubtypic antibody and is stabilized by a hydrogen bond formed between Ser159 and an amino acid of this antibody. A substitution at position 158 or 159 could have an impact on antibodies recognition (Lee et al., 2012).

Most of the EIV strains circulating in Europe in recent years belong to Florida Clade 2 (Bryant et al., 2009; Gildea et al., 2011). This is supported by our study, which indicates that strains isolated from French outbreaks since 2005 were also located within the Florida Clade 2 cluster, with the exception of a large-scale outbreak in 2009 that belongs to Florida Clade 1 (Legrand et al., 2013).

Since 2010, the OIE-ESP recommends the incorporation of representative EIV strains from both Florida Clade 1 and Clade 2 into EI vaccines (OIE World Organisation for Animal Health, 2010). Comparison of HA sequences highlight several substitutions between the French EIV strains and the OIE-recommended strain A/equine/Richmond/1/2007 (Florida Clade 2), such as amino acid substitutions P103L, A144V, I282V, V300I and I527V. The strain A/equine/Saone-et-Loire/1/2015 can be of great interest as this strain presents ten substitutions when compared to A/equine/Richmond/1/07, with three of which (A144T, T192K and Q197K) are located in antigenic sites (site A for the first residue and site B for the other two) with one close to the antigenic site A (T131N). Accumulation of these amino acid substitutions within the antibody-binding sites in the HA could be sufficient to lead to the antigenic drift phenomenon. According to the study conducted by Wilson and Cox (1990), four or five amino acid substitutions in at least two separate antigenic sites were sufficient for escape from pre-existent immunity and lead to vaccine updates for human influenza A viruses. Moreover, previous studies have shown that, in equine influenza A, 10–16 amino acid differences between outbreak and vaccine strains could lead to a vaccine breakdown (Ito et al., 2008; Woodward et al., 2015).

The analysis of other viral components showed the presence of several amino acid substitutions in the polymerase complex (PB2, PB1 and PA), especially for the strains A/equine/Belfond/6-2/2009 and A/equine/Saone-et-Loire/1/2015. A/equine/Belfond/6-2/2009 was located within the Florida Clade 1 cluster. The amino acid substitutions identified in A/equine/Belfond/6-2/2009 were coherent with results obtained by Alves Beuttemmüller et al. (2016), with 21 out of 32 amino acids substitution shared with the Florida Clade 1A/equine/Kentucky/1/2011, A/equine/Dubai/1/2012 and A/equine/Rio Grande do Sul/1/2012 strains.

However, it is surprising to observe such an important number of substitutions in the polymerase complex for the French strain isolated in 2015. Indeed, A/equine/Saone-et-Loire/1/2015 possessed 3, 13 and 11 amino acid substitutions in PB2, PB1 and PA, respectively, compared to other French strains belonging to the Florida Clade 2 cluster (2, 2 and 6 substitutions for A/equine/Jouars/4/2006; 2, 2 and 4 substitutions for A/equine/Neuville-Pres-Sees/1/2011; 1, 1 and 4 substitutions for A/equine/Cambremer/1/2012; and 5, 5 and 6 sub-

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                                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                                    5         15         25         35         45         55         65         75         85
A/equine/Belfond/6-2/2009       MEQGQDTPWI LSTEHTNIQK KGNGQQTLRL EHHNLIQSMD HFLKTMNQVG TPKQIVYWKQ WLYLKNPIPE SLKIRVLKRW R---------
A/equine/Neuville-Pres-Sees/1/2011 ...E..... ......... R......... ......... .........V ......... ..S..... ........Q. R----------
A/equine/Cambremer/1/2012       ...E..... ......... R......... ......... .........V ......... ..S..... ....H...Q. R---------
A/equine/Gironde/1/2014         ...E..I... Q.......R. R......... .Y........ .........V ......... ..S..... ........Q. R---------
A/equine/Saone-et-Loire/1/2015  ...E..... ......... ......... ......... .........V ......... ..S.....L. ....H...Q. RWFSRQEWTN
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Fig. 4. Comparison of PB1-F2 amino acid sequences for French strains isolated since 2009. The strain A/equine/Belfond/6-2/2009 belonging to Florida sub-lineage Clade 1 while the other strains belonging to Florida sub-lineage Clade 2.

Gene	PB2									PB1				PA								PA-X		PB1-F2	M2			NS1
Amino acid position	63	212	295	398	503	660	667	686	731	94	203	584	621	98	259	348	354	409	465	538	636	98	240	63	59	85	87	66
A/equine/Ohio/1/2003	I	I	V	I	R	K	V	V	I	F	R	R	K	T	P	L	T	S	I	E	V	T	A	S	L	D	E	E
A/equine/Belfond/6-2/2009	V	V	I	V	K	R	I	I	V	L	K	Q	R	A	S	I	I	N	V	K	I	A	D	Y	M	G	D	K

Fig. 5. Amino acid differences between the French strain isolated in 2009 and the OIE-recommended vaccine strain A/equine/Ohio/1/2003.

stitutions for A/equine/Gironde/1/2014). The sequence analysis of polymerase complex protein reveals the presence of potential factors of pathogenicity when compared to other Florida Clade 2 strains. Indeed, a previous study carried out by Song et al. (2015) identified two substitutions at position 224 and 383 in PA that appear to alter the viral pathogenicity and viral replication of avian H5N1 viruses in a mouse model. Furthermore, the N383D substitution is associated with an increased polymerase activity in both avian and human cells. Interestingly, all EI strains characterised here exhibit an aspartic acid at position 383 but also the occurrence of S224P substitution for the strain A/equine/Saone-et-Loire/1/2015. Those substitutions which might enhance the pathogenicity and viral replication of EIV should be monitored in future EIV characterisation.

A recent study showed the existence of a pro-inflammatory motif in PB1-F2 from the H3N2 subtype (Alymova et al., 2011). Amino acid residues L62, R75, R79, and L82 (C-terminus region of PB1-F2) from H3N2 influenza A viruses were sufficient to generate an inflammatory response. Mutations at these four positions in the C terminus region of PB1-F2 were sufficient to abrogate PB1-F2 pro-inflammatory activity and lead to pathogenicity attenuation. As a result, it was suggested that some PB1-F2 non-inflammatory motifs (P62, H75, Q79 and S82) may mitigate the risk of secondary bacterial infection (Alymova et al., 2011). Moreover, it has been shown that the PB1-F2 pro-inflammatory motif increased morbidity induced by primary viral infection and enhanced development of secondary bacterial pneumonia in mice. Our study highlights a potential pro-inflammatory motif for the strain A/equine/Belfond/6-2/2009 (FC1; amino acid residues L62, R75 and R79) when compared to the strains A/equine/Cambremer/1/2012 and A/equine/Saone-et-Loire/1/2015 (both FC2), which possess a motif close to the non-inflammatory motif (amino acid residues L62, H75, Q79 and supplementary residue W82 for the A/equine/Saone-et-Loire/1/2015 strain). The strains A/equine/Neuville-Pres-Sees/1/2011 and A/equine/Gironde/1/2014 have an intermediate profile with amino acid residues L62, R75 and Q79. This transition in amino acid sequence within the pro-inflammatory motif was described for a H3N2 strain by Alymova et al. (2011). This transition was first identified in 1972 with an L82S substitution, followed in 1974 by R79Q, in 1987 by R75H, and in 1995 by L62P. These mutations were associated with a decrease in the severity of the clinical outcomes over several decades (Doshi, 2008; Fleming and Elliot, 2008). Our results obtained for the French strains, follow the same transition pattern with the occurrence of substitutions R79Q and R75H in 2011 and 2012, respectively. Moreover, our observations on PB1-F2 were consistent with a suspected decreased pathogenicity observed with recent Florida Clade 2. After experimental infection with the A/equine/Cambremer/1/2012 strain, ponies displayed less severe clinical signs of disease, when compared to infection with the EIV strain A/equine/Richmond/1/2007 (Garrett et al., 2016) (Paillot, personal communication). This is in contrast with the 2009 FC1 EIV strains (i.e. A/equine/Belfond/6-2/2009 and A/equine/Grosbois/13/2009) that caused strong morbidity and had also affected vaccinated horses. The pathogenic EIV strain A/equine/Sussex/1/1989 (Binns et al., 1993; Livesay et al., 1993) also exhibited a motif similar to the pro-inflammatory motif (data not shown). The P69L substitution in A/equine/Saone-et-Loire/1/2015 PB1-F2 is also to be taken into consideration since this isolate presents an isoleucine at position 68 and a glutamic acid in position 70. Indeed, Alymova et al. (2014a), (2014b) recently highlighted a unique motif (I68, L69 and V70) promoting the peptide's cytotoxicity and permeabilisation of the mitochondrial membrane. Moreover, A/equine/Saone-et-Loire/1/2015 PB1-F2 length is increased when compared to EIV strains characterised recently (i.e. 90 amino acids instead of 81 amino acids). Full-length versions of PB1-F2 (predominantly 87 or 90 amino acids) specifically translocate into mitochondria, through their C-terminal region, which acts as a mitochondrial targeting sequence, and induces apoptosis (Yoshizumi et al., 2014).

5. Conclusion

The EIV strains responsible for the EI outbreaks in France since 1967 were relatively close to viruses circulating in Europe at the time. This study reports the first introduction of viruses belonging to Eurasian and American lineages in France in 1999. A predominance of EIV strains belonging to the Florida Clade 2 was recorded since 2005 although the presence of Clade 1 was observed in 2009. The last cases of EIV registered in France (2012–2015) were mainly associated with horse movement and importation. The French EIV strain isolated in 2015 showed 3 amino acid substitutions in 2 separate antigenic sites. These substitutions may increase the risk of vaccine breakdown. However, it is unclear whether these substitutions will remained. This strain also appears to be of interest due to the presence of pathogenicity markers in PA and two motifs similar to the non-inflammatory and cytotoxic motifs in PB1-F2. The results obtained would suggest that monitoring these particular substitutions is important in order to anticipate the potential impact of future EIV antigenic drift on vaccine protection (primarily HA and NA). Furthermore, monitoring the potential novel virulence and transmission determinants in other genes could provide additional information in determining and preventing possible future EIV outbreaks.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2017.02.003.

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