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Emergence of novel equine arteritis virus (EAV) variants during persistent infection in the stallion: Origin of the 2007 French EAV outbreak was linked to an EAV strain present in the semen of a persistently infected carrier stallion

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ABSTRACT

During the summer of 2007, an outbreak of equine viral arteritis (EVA) occurred in Normandy (France). After investigation, a link was suggested between an EAV carrier stallion (A) and the index premise of the outbreak. The full-length nucleotide sequence analysis of a study reference strain (F27) isolated from the lung of a foal revealed a 12,710 nucleotides EAV genome with unique molecular hallmarks in the 5′UTR leader sequence and the ORF1a sequence encoding the non-structural protein 2. The evolution of the viral population in the persistently infected Stallion A was then studied by cloning ORFs 3 and 5 of the EAV genome from four sequential semen samples which were collected between 2000 and 2007. Molecular analysis of the clones confirmed the likely implication of Stallion A in the origin of this outbreak through the yearly emergence of new variants genetically similar to the F27 strain.

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Introduction

Equine arteritis virus (EAV) is the causative agent of equine viral arteritis (EVA), and a member of the family *Arteriviridae* within the order *Nidovirales* (Cavanagh, 1997; Snijder and Spaan, 2006). EVA is a reproductive and respiratory disease in horses and other equid species (Doll et al., 1957a, 1957b; McCollum and Swerczek, 1978). The genome is a positive-stranded 3′-polyadenylated RNA of approximately 12.7 kb in length, with ten known open reading frames flanked by the 5′ untranslated regions (5′UTR) and 3′UTR (Firth et al., 2011; Snijder and Meulenberg, 1998; Snijder et al., 1999). The 5′-terminal leader sequence is an untranslating sequence present in the 5′-proximal region of all genomic and subgenomic mRNAs (van den Born et al., 2005). The 5′-three-quarters of the genome (ORFs 1a and 1b) encode two replicase polyproteins, which are post-translationally processed by three ORF1a-encoded proteinases (nsp1, -2 and -4) in order to yield at least 13 non-structural proteins (nsp1 to -12, including nsp7α and 7β) (Snijder and Meulenberg, 1998; van Aken et al., 2006; van Dinten et al., 1996; Ziebuhr et al., 2000). The remaining 3′-quarter contains eight ORFs which encodes eight structural proteins: E, GP2, GP3, GP4, 5a, GP5, M and N respectively (de Vries et al., 1992; Snijder and Spaan, 2006; Wieringa et al., 2002). The envelope contains two major viral proteins: the unglycosylated membrane protein M and the variable major envelope glycoprotein GP5 which confers different neutralization phenotypes (Balasuriya et al., 1999, 2001; Hedges et al., 1999). Consequently, the ORF 5 is the most widely used for phylogenetic analysis, and global EAV strains can be grouped into two clades: the North American group and the European group which can be divided into two different subgroups named EU-1 and EU-2 (Balasuriya et al., 1995, 1999; Larsen et al., 2001; Metz et al., 2011; Mittelholzer et al., 2006; Pronost et al., 2010; Zhang et al., 2007, 2010).

Exposure to EAV usually results in a mild or subclinical infection in immunocompetent animals. Clinical signs of EAV infected horses can vary in range and severity, and the vast majority of these EAV infections are unapparent. Equine viral arteritis is frequently characterized by influenza-like signs in adult horses, but can also cause abortion in pregnant mares, interstitial pneumonia in young foals and death in newborn foals (McCollum et al., 1999; Timoney and McCollum,

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1993). A variable percentage (up to 10%–70%) of the stallions acutely infected with EAV can subsequently become carriers and continue to shed the virus in their semen. Carrier stallions are the natural reservoir of EAV and the virus persists in the reproductive tract, principally in the ampulla of the vas deferens (Timoney and McCollum, 1993). They are central to the epidemiology of the disease and they can potentially transmit the virus to susceptible mares during artificial insemination or natural breeding (Timoney, 1986; Timoney et al., 1986).

During the summer of 2007, an EAV outbreak occurred in the lower and upper regions of Normandy, France. Only draught and saddle horses were affected on 18 infected premises. This study is based on preliminary investigation of a known long-term EAV carrier stallion and his link with the index premise of the outbreak. Therefore, we worked on the hypothesis that the source of the 2007 EVA outbreak in France stemmed from an EAV strain present in the semen of this carrier stallion. The primary objective of the study was to undertake molecular characterization of the EAV strain associated with this outbreak and to compare its molecular hallmarks to the virus present in the semen of the carrier stallion in order to confirm the origin of this EVA outbreak. A second objective was to study the genetic evolution of the virus present in this stallion from 2000 to 2007.

Results

Retrospective investigation

In 2000, screening of serum samples from a stallion, named Stallion A and located in a Normandy stud farm (SF-A), was diagnosed seropositive for antibodies to EAV. This stallion was then confirmed in 2001 as a chronic EAV shedder by demonstrating viral nucleic acids in semen by standard RT-PCR. On April 6th and 28th, 2007, a Mare B was brought to the Index Premise 61 named IP61 for two separate artificial inseminations with two samples of fresh-cooled semen collected in 2007 from Stallion A (Fig. 1). Upon returning to her stud farm (SF-B), Mare B then became ill after the first insemination of April 11th, 2007, and the EAV infection was confirmed by serological testing. During both artificial inseminations at the index premise (IP61), Mare B was in close contact with a teaser horse, Stallion C, which became infected after the second artificial insemination and was declared ill on May 6th, 2007. Then Stallion C remained at IP61 in close contact with four other stallions (D, E, F and G). On June 25th, 2007, the IP61 was confirmed EAV infected and officially designated as the onset of the outbreak following the death of Stallion D. Between May 16th and June 27th, 2007, fresh-cooled semen from the four Stallions D, E, F and G was sent for artificial insemination to the Primary Premises PP27 and PP61-1. The first established case of mortality due to EAV was observed at the PP61-1 in a very young foal (R), and is considered in this study as the "study reference EAV strain" named F27.

Characterization of nucleotide hallmarks of the F27 EAV strain circulating during the 2007 French EVA outbreak

The full-length of the F27 "study reference strain" is 12,710 nucleotides (nt) in length, 2 nucleotides longer than the CW96 strain (12,708 nt, EU-2 phylogenetic subgroup, GenBank ID AY349167), and 6 nucleotides longer than the EAV030 reference virus (12,704 nt, North American phylogenetic group (NA), GenBank ID Y07862) (Balasuriya et al., 2004; van Dinten et al., 1997). The F27 strain had a 87.0% and 86.0% nucleotide identity on full genome sequences compared respectively to the CW96 and EAV030 strains. The greatest variations among the structural proteins were observed in the glycoproteins GP5, GP3, and GP4, which presented respectively a 85.5%, 85.8% and 87.1% nucleotide identity compared to the EAV030 strain. The greatest variations among the replicase proteins, occurred

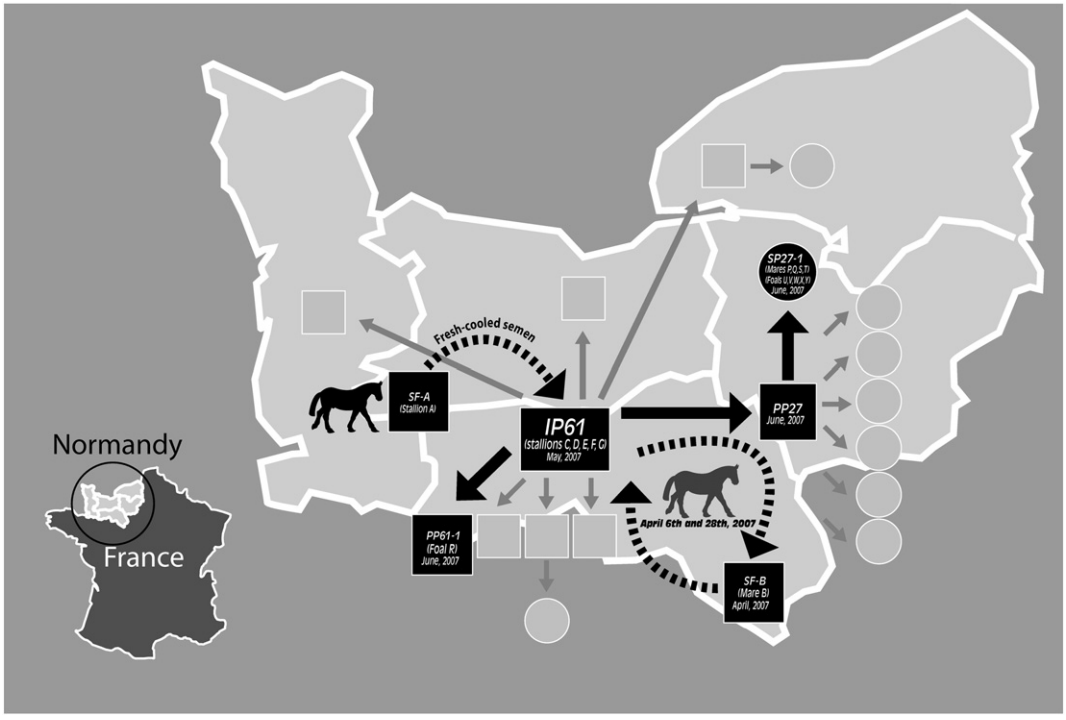

Fig. 1. Schematic depiction of the location of premises to which the EAV infection spreads in Normandy, France. This region is divided in five different areas in Upper (27: Eure, 76: Seine Maritime) and Lower Normandy (14: Calvados, 50: Manche, 61: Orne). Links between the different premises are represented by full grey and black arrows, and fresh-cooled semen and animal movements concerning Stallion A and Mare B (with dates of artificial inseminations) are in black broken arrows. The black squares represent the premises from which samples were collected, and squares and circles in grey represent respectively primary and secondary premises that were subsequently infected, with date of EAV transmission (month, year). The stud farms A and B are named SF-A and SF-B, the index premise is named IP, the 8 primary premises (PP) and the 9 secondary premises (SP), followed by the corresponding area's number.

in the nsp3, nsp2 and nsp7 proteins, which presented respectively a 80.8%, 81.8% and 83.1% nucleotide identity, compared to the published sequence of the EAV030 strain.

The F27 full-length sequence revealed four insertions and one deletion in the genome: one 2-nucleotide insertion (nt numbers T-119 and T-120), two 1-nucleotide insertions (T-156 and C-160) and a single 1-nucleotide deletion (G-138) in its 5′UTR leader sequence, as well as a consecutive 3-nucleotide insertion (nt G-1458, G-1459, C-1460) in a highly variable region of the nsp2 gene. Complementary molecular analyses were performed on the 5′UTR leader and nsp2 regions with respectively 54 and 51 sequences collected from the GenBank database and in this study. The analysis revealed that the 2-nucleotide insertion (T-119 and T-120) had never been described in scientific literature, unlike the two 1-nucleotide insertions and the single deletion in the 5′ leader sequence which had ever been observed in previous studies (Fig. 2A) (Balasuriya et al., 2004; Van Den Born et al., 2004). In the analysis of nsp2 sequences, collected sequences were as follow: 28 sequences collected from GenBank database and 23 sequences from strains of this study (4 strains from Stallion A (F60-F63), 11 strains collected during the outbreak (F27-F67), and 8 before the outbreak between 2001 and 2006 (F10, F15, F68-F73)) (Fig. 2B). Of the 54 sequences, 25 belonged to the EU-2 subgroup, 23 to the EU-1 subgroup and 6 to the North American group. The consecutive 3-nucleotide insertion in position nt 158 to 160 was only observed in sequences from strains belonging to the EU-2 subgroup and was absent in the other clades. Sequences obtained from strains collected from Stallion A and during this 2007 EAV outbreak revealed a specific “GGC” insertion resulting in the insertion of a glycine [G] in position 152 in the nsp2 amino acid sequence. At the same position, 8 sequences from strains collected in France between 2001 and 2006 were characterised by a “GAC” insertion encoding an aspartic acid [D], and 2 previously published sequences (CW96 and CW01) collected from GenBank revealed a “GTC” insertion encoding a valine [V] (Balasuriya et al., 2004).

Characterization of EAV strains from Stallion A

Stallion A was known since 2000 as being EAV positive. The four strains collected from this stallion between 2000 and 2007 (F60-F63) were subjected to the full-length genome sequencing and they were respectively 12,710 nucleotides in length (F60), and 12,722 nucleotides (F61, F62, F63). Analysis of these sequences revealed the same insertions and deletion in the 5′UTR leader and nsp2 sequences of those observed in the F27 sequence. Chromatograms of these sequences

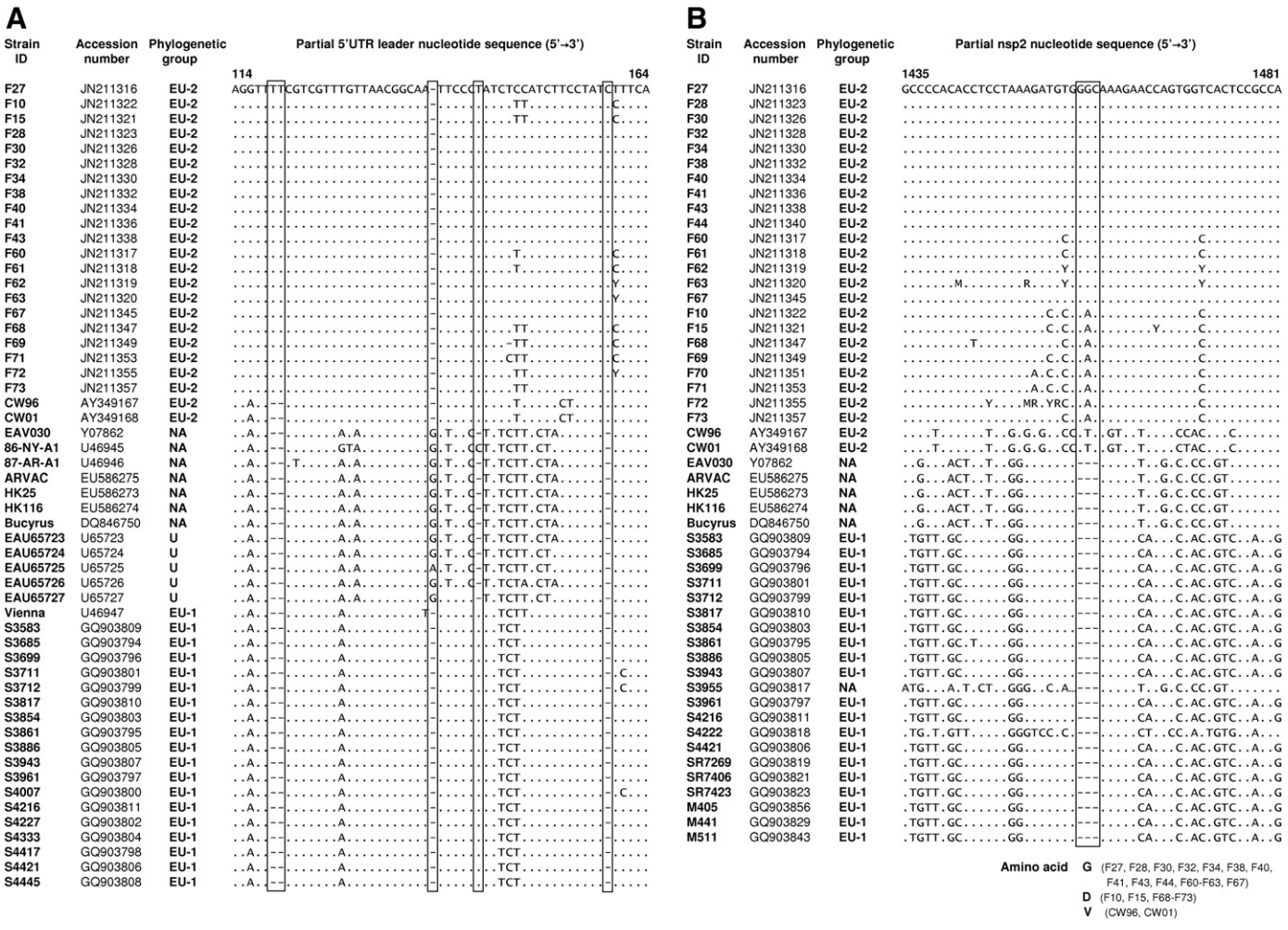

Fig. 2. Alignment and nucleotide variations of partial nucleotide sequences of the 5′UTR leader region (A) and the nsp2-coding region in ORF1a (B). Respectively 54 and 51 sequences of the 5′UTR leader and the nsp2 regions were aligned, collected from the GenBank database and this study (F10, F15, F27, F28, F30, F32, F34, F38, F40, F41, F43, F44, F60-F63, F67-F73) (first and second columns). Alignments were performed using Vector NTI Advance® v11.5.1 (Invitrogen-Life Technologies, Carlsbad, CA). The third column corresponds to the phylogenetic group (NA, North American group; EU-1, European subgroup 1; EU-2, European subgroup 2; U, Undetermined) (Pronost et al., 2010; Zhang et al., 2007, 2010). The nucleotides were numbered according to their position in the published sequence of EAV030 virus (GenBank accession no. Y07862) (van Dinten et al., 1997). Panel A: frameworks correspond to insertions and deletion in the sequences collected in this study, compared to the EAV030 reference sequence. Panel B: framework corresponds to insertion in the sequences collected in this study, compared to the EAV030 reference sequence. Amino acids are translated from the 3-nucleotide insertions (G, glycine; D, aspartic acid, V, valine) and corresponding strains.

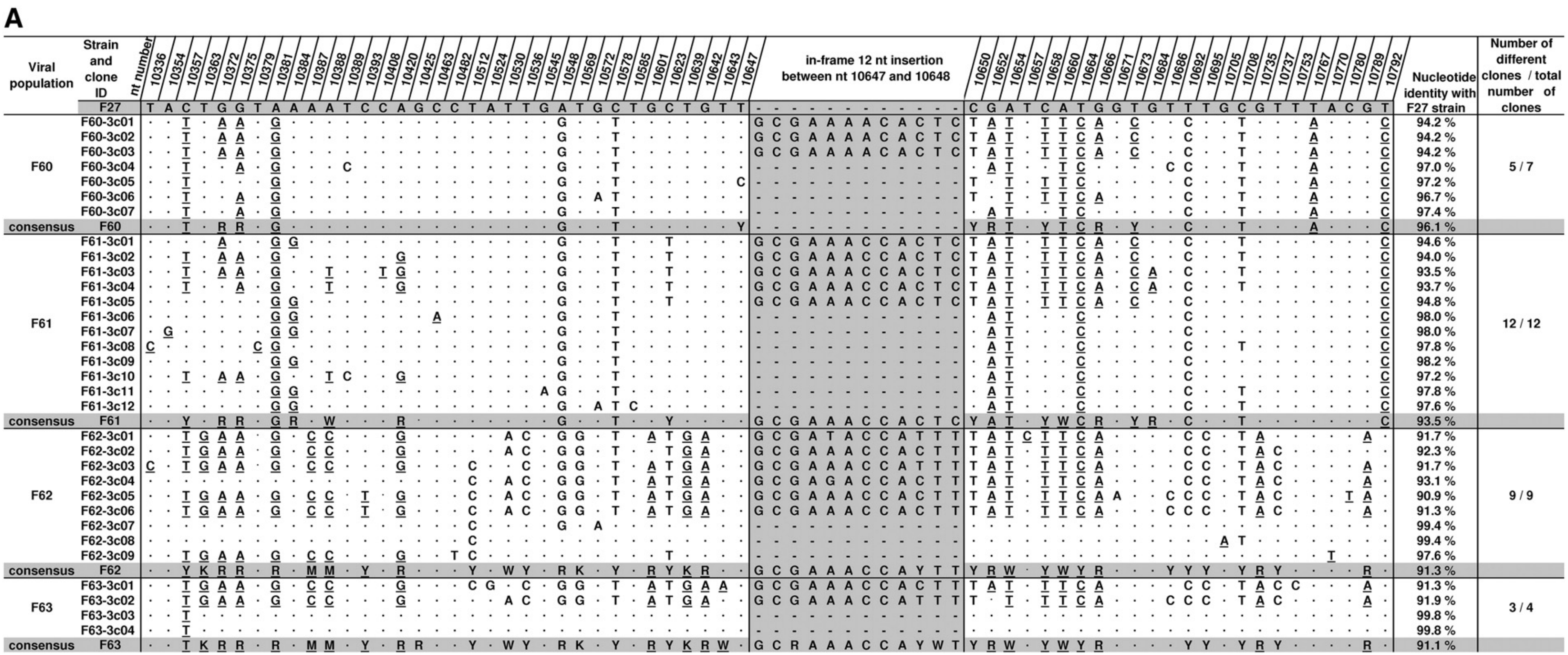

Fig. 3. Nucleotide variations observed on ORF3 (A) and ORF5 (B) in the different clones obtained from F60, F61, F62 and F63 viral populations (Stallion A) and compared to the study reference F27 sequence. Clones are named by viral population (F60 to F63), then by cloned ORF (-3 or -5) and then by number of clone in the studied viral population (c#). The nucleotides were numbered according to their position in the published sequence of EAV030 virus (GenBank accession no. Y07862) (van Dinten et al., 1997). Only differences were scored. Dots indicate sequence identity. Variations in the F27 sequence, consensus sequences of F60 to F63 viral populations and in-frame 12-nucleotide insertion in ORF3 are in grey. Non-silent nucleotide mutations, compared to the F27 sequence, are underlined. The number of different clones was determined by comparison with the total number of obtained clones in each viral population (F60, F61, F62 and 63).

B

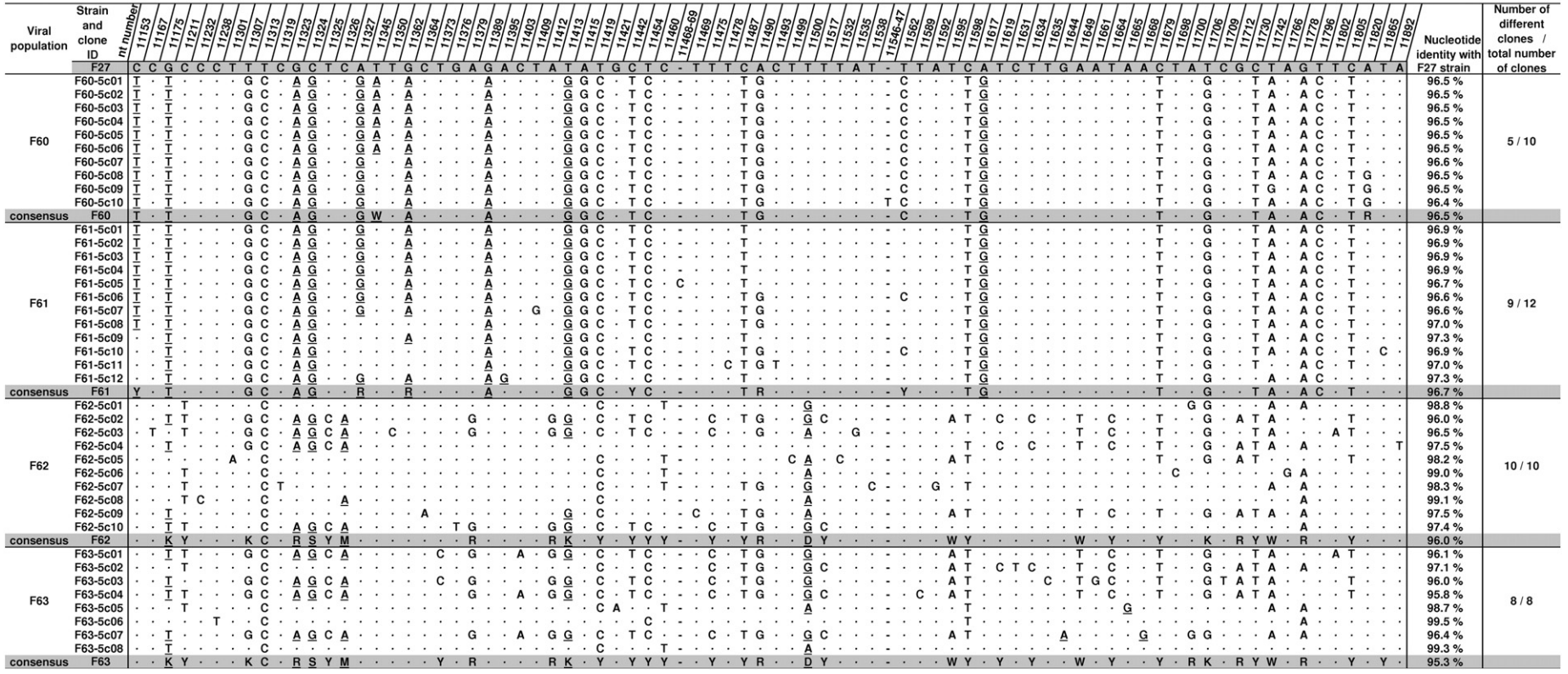

Fig. 3 (*continued*).

A

Viral population	Strain and clone ID	aa number 11	17	18	20	23	24	25	26	27	28	30	35	39	53	99	112	113	in-frame 4 amino acids insertion	116	117	118	119	120	121	123	127	134	144	154	155	159	162	163	Amino acid identity with F27 strain	Number of different clones / total number of clones
	F27	F	N	L	F	G	G	G	S	S	N	H	H	N	P	R	L	V	- - - -	R	T	S	T	I	E	L	A	D	F	F	L	R	G	S	
F60	F60-3c01	·	·	F	·	R	S	·	G	·	·	·	·	·	·	·	·	·	A K T L	Q	S	L	S	T	K	S	·	·	·	L	·	·	·	P	89.9 %	4 / 7
	F60-3c02	·	·	F	·	R	S	·	G	·	·	·	·	·	·	·	·	·	A K T L	Q	S	L	S	T	K	S	·	·	·	L	·	·	·	P	89.9 %	
	F60-3c03	·	·	F	·	R	S	·	G	·	·	·	·	·	·	·	·	·	A K T L	Q	S	L	S	T	K	S	·	·	·	L	·	·	·	P	89.9 %	
	F60-3c04	·	·	F	·	·	S	·	G	·	·	·	·	·	·	·	·	·	- - - -	Q	S	·	S	T	·	·	·	·	·	L	·	·	·	P	94.5 %	
	F60-3c05	·	·	F	·	·	·	·	G	·	·	·	·	·	·	·	·	·	- - - -	·	S	L	S	T	·	·	·	·	·	L	·	·	·	P	95.1 %	
	F60-3c06	·	·	F	·	·	S	·	G	·	·	·	·	·	·	·	·	·	- - - -	·	S	L	S	T	K	·	·	·	·	L	·	·	·	P	93.9 %	
	F60-3c07	·	·	F	·	·	S	·	G	·	·	·	·	·	·	·	·	·	- - - -	Q	S	·	S	T	·	·	·	·	·	L	·	·	·	P	94.5 %	
consensus	F60	·	·	F	·	X	X	·	G	·	·	·	·	·	·	·	·	·	- - - -	X	S	X	S	T	X	X	·	·	·	L	·	·	·	P	90.9 %	
F61	F61-3c01	·	·	·	·	·	·	·	G	G	·	·	·	·	·	·	·	·	A K P L	Q	S	L	S	T	K	S	·	·	·	·	·	·	·	P	91.7 %	9 / 12
	F61-3c02	·	·	F	·	R	S	·	G	·	·	·	·	D	·	·	·	·	A K P L	Q	S	L	S	T	K	S	·	·	·	·	·	·	·	P	89.9 %	
	F61-3c03	·	·	F	·	R	S	·	G	·	I	·	Y	D	·	·	·	·	A K P L	Q	S	L	S	T	K	S	T	·	·	·	·	·	·	P	88.1 %	
	F61-3c04	·	·	F	·	R	S	·	G	·	I	·	·	D	·	·	·	·	A K P L	Q	S	L	S	T	K	S	T	·	·	·	·	·	·	P	88.7 %	
	F61-3c05	·	·	·	·	·	·	·	G	G	·	·	·	·	·	·	·	·	A K P L	Q	S	L	S	T	K	S	·	·	·	·	·	·	·	P	91.7 %	
	F61-3c06	·	·	·	·	·	·	·	G	G	·	·	·	·	Q	·	·	·	- - - -	Q	S	·	·	T	·	·	·	·	·	·	·	·	·	·	P	95.7 %	
	F61-3c07	·	D	·	·	·	·	·	G	G	·	·	·	·	·	·	·	·	- - - -	Q	S	·	·	T	·	·	·	·	·	·	·	·	·	·	P	95.7 %	
	F61-3c08	L	·	·	·	·	·	A	G	·	·	·	·	·	·	·	·	·	- - - -	Q	S	·	·	T	·	·	·	·	·	·	·	·	·	·	P	95.7 %	
	F61-3c09	·	·	·	·	·	·	·	G	G	·	·	·	·	·	·	·	·	- - - -	Q	S	·	·	T	·	·	·	·	·	·	·	·	·	·	P	96.3 %	
	F61-3c10	·	·	F	·	R	S	·	G	·	I	·	·	D	·	·	·	·	- - - -	Q	S	·	·	T	·	·	·	·	·	·	·	·	·	·	P	93.9 %	
	F61-3c11	·	·	·	·	·	·	·	G	G	·	·	·	·	·	·	·	·	- - - -	Q	S	·	·	T	·	·	·	·	·	·	·	·	·	·	P	96.3 %	
	F61-3c12	·	·	·	·	·	·	·	G	G	·	·	·	·	·	·	·	·	- - - -	Q	S	·	·	T	·	·	·	·	·	·	·	·	·	·	P	96.3 %	
consensus	F61	·	·	X	·	X	X	·	G	X	X	·	·	X	·	·	·	·	A K P L	Q	S	X	X	T	X	X	X	·	·	·	·	·	·	·	P	86.9 %	
F62	F62-3c01	·	·	F	V	R	S	·	G	·	P	·	·	D	·	H	V	I	A I P F	Q	S	L	S	T	K	·	·	·	I	·	·	·	S	·	86.9 %	9 / 9
	F62-3c02	·	·	F	V	R	S	·	G	·	P	·	·	D	·	·	V	I	A K P L	Q	S	L	S	T	K	·	·	·	I	·	·	·	·	·	88.1 %	
	F62-3c03	L	·	F	V	R	S	·	G	·	P	·	·	D	·	H	V	I	A K P F	Q	S	L	S	T	K	·	·	·	I	·	·	·	S	·	86.3 %	
	F62-3c04	·	·	·	·	·	·	·	·	·	·	·	·	·	·	H	V	I	A R P L	Q	S	L	S	T	K	·	·	·	I	·	·	·	S	·	91.1 %	
	F62-3c05	·	·	F	V	R	S	·	G	·	P	Y	·	D	·	H	V	I	A K P L	Q	S	L	S	T	K	·	·	·	I	·	·	C	S	·	85.7 %	
	F62-3c06	·	·	F	V	R	S	·	G	·	P	Y	·	D	·	H	V	I	A K P L	Q	S	L	S	T	K	·	·	·	I	·	·	·	S	·	86.3 %	
	F62-3c07	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	- - - -	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	100.0 %	
	F62-3c08	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	- - - -	·	·	·	·	·	·	·	·	N	·	·	·	·	·	·	·	99.4 %	
	F62-3c09	·	·	F	V	R	S	·	G	·	P	·	·	D	·	·	·	·	- - - -	·	·	·	·	·	·	·	·	·	·	F	·	·	·	·	95.1 %	
consensus	F62	·	·	X	X	X	X	·	X	·	X	X	·	X	·	X	X	X	A K P X	X	X	X	X	X	X	·	X	·	X	·	·	·	X	·	79.2 %	
F63	F63-3c01	·	·	F	V	R	S	·	G	·	P	·	·	D	·	H	V	N	A K P L	Q	S	L	S	T	K	·	·	·	I	·	·	·	S	·	86.9 %	3 / 4
	F63-3c02	·	·	F	V	R	S	·	G	·	P	·	·	D	·	H	V	I	A K P F	·	S	L	S	T	K	·	·	·	I	·	·	·	S	·	87.5 %	
	F63-3c03	·	·	F	·	·	·	·	·	·	·	·	·	·	·	·	·	·	- - - -	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	99.4 %	
	F63-3c04	·	·	F	·	·	·	·	·	·	·	·	·	·	·	·	·	·	- - - -	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	99.4 %	
consensus	F63	·	·	F	X	X	X	·	X	·	X	X	·	X	·	X	X	X	A K P X	X	X	X	X	X	X	·	·	·	X	·	·	·	X	·	79.2 %	

B

Viral population	Strain and clone ID	aa number 3	10	60	61	67	73	82	84	90	119	158	167	174	175	Amino acid identity with F27 strain	Number of different clones / total number of clones
	F27	S	L	A	Q	V	V	D	I	L	F	I	G	I	I		
F60	F60-5c01	F	F	S	R	E	I	N	·	V	·	V	·	·	·	96.5 %	2 / 10
	F60-5c02	F	F	S	R	E	I	N	·	V	·	V	·	·	·	96.5 %	
	F60-5c03	F	F	S	R	E	I	N	·	V	·	V	·	·	·	96.5 %	
	F60-5c04	F	F	S	R	E	I	N	·	V	·	V	·	·	·	96.5 %	
	F60-5c05	F	F	S	R	E	I	N	·	V	·	V	·	·	·	96.5 %	
	F60-5c06	F	F	S	R	E	I	N	·	V	·	V	·	·	·	96.5 %	
	F60-5c07	F	F	S	R	·	I	N	·	V	·	V	·	·	·	96.9 %	
	F60-5c08	F	F	S	R	·	I	N	·	V	·	V	·	·	·	96.9 %	
	F60-5c09	F	F	S	R	·	I	N	·	V	·	V	·	·	·	96.9 %	
	F60-5c10	F	F	S	R	·	I	N	·	V	·	V	·	·	·	96.9 %	
consensus	F60	F	F	S	R	X	I	N	·	V	·	V	·	·	·	96.1 %	
F61	F61-5c01	F	F	S	R	·	I	N	·	V	·	V	·	·	·	96.9 %	5 / 12
	F61-5c02	F	F	S	R	·	I	N	·	V	·	V	·	·	·	96.9 %	
	F61-5c03	F	F	S	R	·	I	N	·	V	·	V	·	·	·	96.9 %	
	F61-5c04	F	F	S	R	·	I	N	·	V	·	V	·	·	·	96.9 %	
	F61-5c05	F	F	S	R	·	I	N	·	V	·	V	·	·	·	96.9 %	
	F61-5c06	F	F	S	R	·	I	N	·	V	·	V	·	·	·	96.9 %	
	F61-5c07	F	F	S	R	·	I	N	·	V	·	V	·	·	·	96.9 %	
	F61-5c08	F	F	S	·	·	·	N	·	V	·	V	·	·	·	97.7 %	
	F61-5c09	·	F	S	·	·	I	N	·	V	·	V	·	·	·	97.7 %	
	F61-5c10	·	F	S	·	·	·	N	·	V	·	V	·	·	·	98.0 %	
	F61-5c11	·	F	S	·	·	·	N	·	V	·	V	·	·	·	98.0 %	
	F61-5c12	·	F	S	R	·	I	N	V	V	·	V	·	·	·	96.9 %	
consensus	F61	X	F	S	X	·	X	N	·	V	·	V	·	·	·	95.7 %	
F62	F62-5c01	·	·	·	·	·	·	·	·	·	V	·	·	·	·	99.6 %	8 / 10
	F62-5c02	·	F	S	K	·	·	·	·	V	V	·	·	·	·	98.0 %	
	F62-5c03	·	·	S	K	·	·	·	·	V	I	·	·	·	·	98.4 %	
	F62-5c04	·	F	S	K	·	·	·	·	·	·	·	·	·	·	98.8 %	
	F62-5c05	·	·	·	·	·	·	·	·	·	I	·	·	·	·	99.6 %	
	F62-5c06	·	·	·	·	·	·	·	·	·	I	·	·	·	·	99.6 %	
	F62-5c07	·	·	·	·	·	·	·	·	·	V	·	·	·	·	99.6 %	
	F62-5c08	·	·	·	K	·	·	·	·	·	I	·	·	·	·	99.2 %	
	F62-5c09	·	F	·	·	·	·	·	·	V	I	·	·	·	·	98.8 %	
	F62-5c10	·	F	S	K	·	·	·	·	V	V	·	·	·	·	98.0 %	
consensus	F62	·	X	X	X	·	·	·	·	X	X	·	·	·	·	89.1 %	
F63	F63-5c01	·	F	S	K	·	·	·	·	V	·	·	·	·	·	98.0 %	5 / 8
	F63-5c02	·	·	·	·	·	·	·	·	·	·	·	·	·	·	99.6 %	
	F63-5c03	·	F	S	K	·	·	·	·	V	·	·	·	·	·	98.0 %	
	F63-5c04	·	F	S	K	·	·	·	·	V	·	·	·	·	·	98.0 %	
	F63-5c05	·	·	·	·	·	·	·	·	·	·	·	·	V	·	99.2 %	
	F63-5c06	·	·	·	·	·	·	·	·	·	·	·	·	·	·	100.0 %	
	F63-5c07	·	F	S	K	·	·	·	·	V	·	·	S	·	V	97.3 %	
	F63-5c08	·	F	·	·	·	·	·	·	·	·	·	·	·	·	99.2 %	
consensus	F63	·	X	X	X	·	·	·	·	X	·	X	·	·	·	87.1 %	

Fig. 4. Amino acid variations observed on ORF3 (A) and ORF5 (B) in the different clones obtained from F60, F61, F62 and F63 viral populations (Stallion A) and compared to the study reference F27 sequence. Clones are named by viral population (F60 to F63), then by cloned ORF (-3 or -5) and then by number of clone in the studied viral population (c#). The amino acids were numbered according to their position in the published sequence of EAV030 virus (GenBank accession no. Y07862) (van Dinten et al., 1997). Only differences were scored. Dots indicate sequence identity. Variations in the F27 sequence, consensus sequences of F60 to F63 viral populations and in-frame 4-amino acids insertion in ORF3 are in grey. The number of different clones was determined by comparison with the total number of obtained clones in each viral population (F60, F61, F62 and 63).

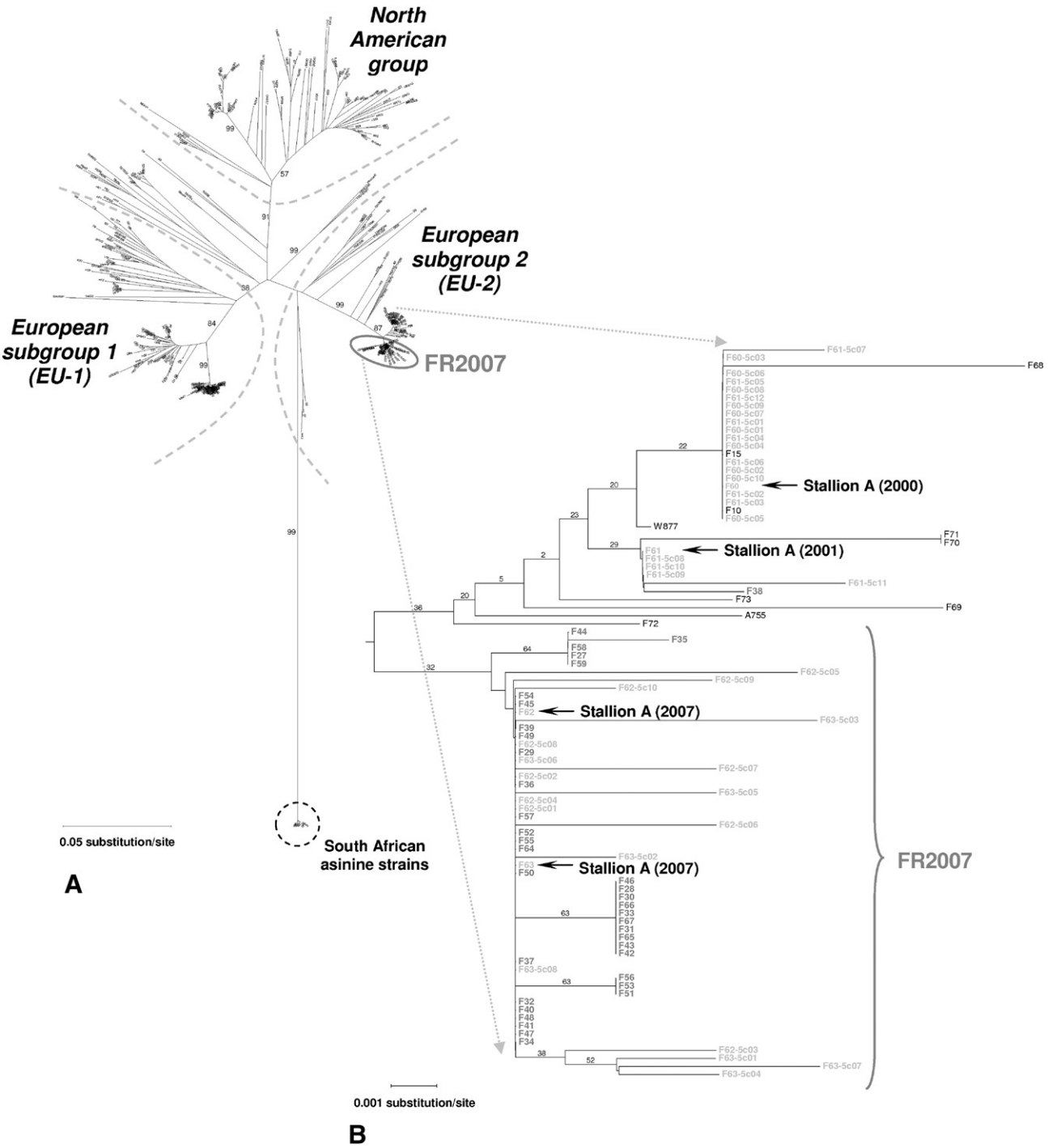

Fig. 5. A) Phylogenetic tree of the partial ORF5 sequences of 388 EAV strains, 66 sequences were collected from this study (26 from EAV strains and 40 clones from F60, F61, F62 and F63 strains) and 322 sequences were previously published (Pronost et al., 2010; Zhang et al., 2007, 2010). B) Enlarged dendrogram of the FR2007 cluster and the phylogenetically closed EAV strains of this cluster. F60 to F63 strains corresponding to consensus sequences from viral populations F60 to F63 collected from Stallion A in 2000, 2001 and 2007, are indicated by arrows. Phylogenetic analyses were constructed using MEGA4.1. Values resulting from bootstrap analysis are indicated adjacent to the major branching points.

revealed some nucleotide positions with ambiguities (various possible nucleotides), which suggests that F60 to F63 full-length genome sequences represent consensus sequences of multiple variants in the same strain. Analysis of the ORF3 and ORF5 sequences of the F60 to F63 strains revealed respectively 8, 13, 34 and 36 ambiguities observed on the ORF3, as well as 2, 6, 31 and 36 ambiguities on the ORF5, which show that the number of ambiguities increased between 2000 and 2007 (Figs. 3A and B). Analysis of full-length genomes also revealed a consecutive in-frame 12-nucleotide insertion in the ORF3 sequence of the F61, F62 and F63 consensus sequences, which was unobserved in other strains and unfound in scientific literature. In these F61 to F63 consensus sequences, the 12-nucleotide insertions were located between nt 10647 and 10648 according to EAV030 sequence, and were genetically similar when compared together (F61: 5′GCGAAACCACTC3′, F62: 5′GCGAAACCAYTT3′, F63: 5′GCRAAACCAYWT3′), and resulted in the insertion of 4 amino acids (F61: AKPL, F62: AKP(L/F), F63: AKP(L/H/Y/F)) (Figs. 3A and 4A).

Genetic evolution of strains in the persistently infected Stallion A

In order to study the viral population distribution, PCR products of variable and complete ORF3 (492 bp) and ORF5 (768 bp) sequences

were cloned from F60 to F63 viral populations of Stallion A. 72 clones were obtained: 32 clones on ORF3 and 40 clones on ORF5, they were sequenced, analysed and compared with the F27 sequence as reference. Analysis revealed two 1-nucleotide insertions in ORF5 from the clones F60-5c10 (nt T-11547) and F61-5c05 (nt C-11469), consequently causing a stop codon in the reading frame (Fig. 3B). Molecular analysis of the ORF5 sequences from the clones revealed that the number of genetically different clones inside each viral population F60 to F63 was 5 out of 10 (F60), 9 out of 12 (F61), 10 out of 10 (F62), and 8 out of 8 (F63). Molecular analysis of the ORF3 sequences from the clones F60 to F63 revealed the presence of variants with the in-frame 12-nucleotide insertion between nt 10647 and 10648 (Fig. 3A). Therefore, this 12-nucleotide insertion in the ORF3 was not present in the reference F27 sequence. Regarding the clones derived from ORF3, 5 out of 7 (F60), 12 out of 12 (F61), 9 out of 9 (F62), and 3 out of 4 (F63) were found to be genetically different. These findings indicate that the number of different clones increased from year to year with a more and more heterogeneous viral population. For viral populations F60 and F63 (on ORF3) and F60 and F61 (on ORF5), several clones were identical and would represent a major variant within these viral populations. However, in viral populations F61 and F62 (on ORF3) and F62 and F63 (on ORF5), all clones were unique and no major variant could be distinguished (Figs. 3A and B). Comparison of each clone to the F27 sequence revealed that the genetically closer clones in each viral population (F60-F63) were F60-3c07, F61-3c09, F62-3c07 and -3c08 and F63-3c03 and -3c04 which had respectively a 97.4%, 98.2%, 99.4% and 99.8% nucleotide identity on ORF3. Likewise, on ORF5, the F60-5c07, F61-5c12, F62-5c08 and F63-5c06 clones had respectively a 96.6%, 97.3%, 99.1% and 99.5% nucleotide identity (Figs. 3A and B). This finding reveals that some of the variants of the quasispecies were, on a yearly basis, increasingly genetically closer to the F27 strain. Furthermore, two clones obtained on ORF3 and ORF5 (F62-3c07 and F63-5c06), both collected in 2007, revealed a 100% amino acid identity with the F27 sequence (Figs. 4A and B). Compared to the F27 nucleotide sequence, of the 32 clones obtained on ORF3, 542 mutations were observed: 198 silent mutations and 344 non-silent mutations corresponding to 337 amino acid changes. Of the 40 clones obtained on ORF5, 881 nucleotide mutations were observed, 626 silent mutations and 255 non-silent mutations corresponding to 218 amino acid changes (Figs. 3 and 4).

Phylogenetic analysis of EAV strains related to the 2007 French EVA outbreak and variants obtained from Stallion A

Based on the phylogenetic analysis of 388 nucleotide sequences of the partial ORF5, an unrooted phylogenetic tree was obtained and EAV strains clustered into three distinct groups: the North American group and the two European subgroups EU-1 and EU-2. All EAV strains related to the 2007 French EVA outbreak clustered inside the European subgroup-2 (EU-2) into the FR2007 group (Fig. 5A). Furthermore, an enlarged dendrogram of the cluster FR2007 and strains phylogenetically closed confirmed that the FR2007 cluster includes all of the EAV strains from the outbreak (Fig. 5B). It also includes the F62 and F63 viral populations collected from Stallion A in 2007 with all of the clones obtained from these two strains. Other clones, obtained from the F60 and F61 strains collected in 2000 and 2001, were phylogenetically related to the FR2007 cluster but grouped outside of the cluster, with the eight strains received by the Frank Duncombe Laboratory between 2001 and 2006 and two other strains W877 and A755 collected from horses in Germany in 1994 and 1995.

Discussion

During the French EVA outbreak, the virus subsequently spread to premises in Normandy from mid-June, 2007, and continued until mid-August. The origin of the outbreak had yet to be identified and it had been hypothesized that a persistently infected stallion could be the source of the infection by the emergence of a new variant which might become more pathogenic (Pronost et al., 2010). Based on a retrospective investigation concerning horses implicated in the putative origin of the outbreak, a link has been established between the persistently infected Stallion A and the index premise. In order to confirm whether the stallion is responsible for precipitating this extensive EVA outbreak in France, we performed extensive sequence and phylogenetic analyses of EAV strain present in the semen of this carrier stallion as well as virus strains present in the animals with clinical signs of EVA. Full-length sequencing of the F27 reference study strain revealed an EAV genome 12,710 nt longer than the EAV030 reference strain (12,704 nt), and shorter than EAV strains associated with the US 2006/2007 occurrence (12,731 nt) (Zhang et al., 2010). Furthermore, molecular characterization of the F27 strain and EAV strains associated with the 2007 disease occurrence showed some interesting and characteristic insertions in the 5′UTR leader and the nsp2 sequences. Further analysis revealed that the first insertion of 2 nucleotides (nt positions 119 and 120) in the 5′UTR leader region had never been described in scientific literature or deposited in GenBank, that it is specific to strains circulating in France, and belongs to the EU-2 subgroup. Interestingly, the 3-nucleotide insertion in nsp2 region also constitutes a genetic marker of the European subgroup-2. Moreover, specific insertion of the 3 nucleotides "GGC" encoding for a Glycine represents a molecular hallmark of the strains collected during the 2007 French EAV outbreak. Genetic analysis on consensus sequences F60 to F63 from Stallion A revealed that these two specific molecular hallmarks, observed in the 5′ leader and the nsp2 sequences of the F27 strain, were both present in strains from Stallion A. Furthermore, the increasing number of ambiguities in the consensus sequences of viral populations from F60 to F63 suggests that these consensus sequences represent a more and more heterogeneous mutant swarm. These heterogeneous populations containing genetically non-identical, but related variants, are commonly termed quasispecies, which are characterised by a dynamic evolution under selective pressures. The extension of quasispecies is due to a distribution of sequences and mutation is not occasional but a continuous event, particularly during chronic or persistent infections. Interestingly, mutations can occur in individual components of a mutant spectrum without change in the average or consensus sequence. This is fuel for viral disease emergences, since viral quasispecies may provide more fit or more pathogenic variants ready to be selected and to cause an epidemic (Domingo, 2010).

Several studies had previously shown that the genetic and phenotypic variations of EAV occurred during persistent infection of stallions (Balasuriya et al., 2004; Hedges et al., 1999; Zhang et al., 2010). The investigation on the four viral populations from Stallion A has clearly demonstrated the dynamic evolution of a viral population over a period of 8 years to one or several variants genetically closed to the F27 strain circulating during the outbreak. Phylogenetic analysis has also established the evolution of viral population for Stallion A, where some variants emerged genetically similar to the epidemic F27 strain. Molecular analysis of clones on the ORFs 3 and 5 indicates that EAV quasispecies evolve considerably in the semen of the carrier stallion during a long-term persistent infection. Full-length sequencing of F61 to F63 has revealed a 12-nucleotide insertion in the ORF3 which was not present in the F27 reference sequence, therefore cloning of these F60-F63 viral populations revealed variants without this insertion, genetically similar to the F27 strain. The description of viral quasispecies has often been performed on small amplicons (<0.5 kb) on other viruses; in this study, analysis of these two genomic segments constitutes a longer and sufficient size to increase the analytical power and reveals a greater complexity of viral swarm (Smith et al., 1997). Viral quasispecies constitute a reservoir of genetic and phenotypic variants, with several biological implications for viral evolution and pathogenesis. One sees

that variants are genetically more and more different with time; for instance, inside each quasispecies, some of the variants were, on a yearly basis, increasingly genetically closer to the F27 strain. These findings and the emergence of new variants in Stallion A, where some of which have a 99.8% and 99.5% nucleotide identity with F27 strain on ORF3 and ORF5, strongly indicate that Stallion A is most probably at the origin of this outbreak, particularly as two clones revealed a 100% identity on the amino acids sequence.

This study confirms that EAV viral RNA evolves in persistently infected stallions, and that 5′UTR leader region and nsp2, GP3 and GP5 genes are involved in the variations of the EAV genome. In order to trace the origin of the outbreak, it is vital to carry out sequential analysis, to compare the virus strains and to monitor their propagation. Furthermore, this study confirms the importance of keeping trace of EAV carrier stallions. These stallions constitute the natural reservoir of EAV, which then ensures its persistence in various horse populations throughout the world; they can also be the cause of new infection at the origin of outbreak. These findings reinforce the importance to monitor, and find treatments for EAV persistent carrier stallions. Efforts continuing into devising a safe and effective therapeutic means of eliminating the carrier state in the stallion are necessary and could considerably reduce the transmission of EAV and the emergence of pathogenic strains as a potential source of new outbreaks.

Materials and methods

Samples

This study included 27 clinical samples collected in France over a period of 8 years (2000 to 2007): 4 sequential semen samples collected from the persistently infected Stallion A (2000, 2001 and twice in 2007), 15 samples (4 semen, 6 nasal swabs and 5 organs) from three of the first premises infected (IP61, PP61-1 and SP27-1) and 8 semen samples received at the Frank Duncombe Laboratory between 2001 and 2006 which were selected since they belonged to the EAV European subgroup-2 (EU-2) (Table 1).

Table 1
Background data of 27 French EAV molecular strains collected in this study.

Strain ID	Sample source	Specimen type	Collection date	Premise	GenBank ID
F60	Stallion A	Semen	23-Feb-00	SF-A	JN211317
F61	Stallion A	Semen	29-Mar-01	SF-A	JN211318
F15	Stallion I	Semen	8-Oct-01	Unknown	JN211321; EF492553
F10	Stallion H	Semen	28-Jan-03	Unknown	JN211322; EF492548
F68	Stallion J	Semen	21-Oct-03	Unknown	JN211347; JN211348
F70	Stallion L	Semen	26-Apr-06	Unknown	JN211351; JN211352
F71	Stallion M	Semen	26-Apr-06	Unknown	JN211353; JN211354
F73	Stallion O	Semen	26-Sep-06	Unknown	JN211357
F69	Stallion K	Semen	3-Oct-06	Unknown	JN211349; JN211350
F72	Stallion N	Semen	19-Oct-06	Unknown	JN211355; JN211356
F28	Stallion D	Testis	1-Jun-07	IP61	JN211323; JN211324
F29	Mare P	Nasal swab	5-Jun-07	SP27-1	JN211325
F30	Mare Q	Nasal swab	5-Jun-07	SP27-1	JN211326; JN211327
F27	Foal R	Lung	15-Jun-07	PP61-1	JN211316
F65	Mare S	Nasal swab	19-Jun-07	SP27-1	JN211343
F32	Mare T	Lung	22-Jun-07	SP27-1	JN211328; JN211329
F34	Foal U	Lung	24-Jun-07	SP27-1	JN211330; JN211331
F38	Stallion E	Semen	27-Jun-07	IP61	JN211332; JN211333
F40	Stallion F	Semen	27-Jun-07	IP61	JN211334; JN211335
F41	Stallion G	Semen	27-Jun-07	IP61	JN211336; JN211337
F43	Foal V	Nasal swab	27-Jun-07	SP27-1	JN211338; JN211339
F44	Foal W	Lung	27-Jun-07	SP27-1	JN211340; JN211341
F66	Foal X	Nasal swab	27-Jun-07	SP27-1	JN211344
F64	Stallion C	Semen	27-Jun-07	IP61	JN211342
F67	Foal Y	Nasal swab	6-Jul-07	SP27-1	JN211345; JN211346
F62	Stallion A	Semen	25-Jul-07	SF-A	JN211319
F63	Stallion A	Semen	10-Sep-07	SF-A	JN211320

Strains are arranged by collection date from February 2000 to September 2007 and named non-exhaustively from F10 to F73 ("F" for French strains and numbers according to Pronost et al., 2010). First part of the table (from February 2000 to October 2006) corresponds to strains collected before the outbreak and the second part (from June 2007 to September 2007) corresponds to strains collected during the outbreak. The three premises, IP61: Index Premise 61, PP61-1: Primary Premise 61-1 and SP27-1: Secondary Premise 27-1, are epidemic premises located in Normandy. The four strains (F60-F63) from the persistently infected Stallion A and the "study reference strain" named F27, were all completely sequenced.

RNA extraction

Viral RNA was extracted from fluid samples (semen and nasal swab) using the QIAamp® Viral RNA isolation kit, and from organ samples (lung, liver and testicle) with 10% tissue suspensions using the RNeasy® Mini Kit, according to the manufacturer's instructions (Qiagen, Hilden, Germany).

Reverse transcription (RT) and PCR amplification

In order to cover the full-length of the EAV genome, viral RNA was first reverse-transcribed into cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's instructions, and then amplified by using forward and reverse primers described in Table 2. PCRs were carried out in a final volume of 50 µl containing 2.5 µl of the cDNA, 10 µl of 5× Phusion HF buffer, 1 µl of dNTP mix (10 mM each), 1.2 µl of each primer (20 µM), 0.5 µl of Phusion™ Hot Start High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland), and 33.6 µl of nuclease-free water. The thermal profile included initial denaturation at 98 °C for 30 s, followed by 40 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s–2 min (depending on the final PCR product size), and a final extension at 72 °C for 10 min. Prior to sequencing, PCR products were gel-purified then both sense and anti-sense strands were sequenced (Eurofins MWG Operon, Huntsville, AL).

Cloning

The entire ORFs 3 and 5 of the four semen samples collected from Stallion A (F60-63) were amplified with flanking primers (10142P–10952N and 10763P–11995N) (Table 2). The PCR products were cloned into the pDrive Cloning Vector and transformed into EZ Competent Cells using the Qiagen® PCR Cloning^{plus} Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The nucleotide sequences of the clones obtained in this study were deposited in GenBank under accession numbers JN211358–JN211429.

Table 2
Primers used for Reverse Transcription, PCR amplification and cloning of EAV genome.

Primer	Polarity	Position	Nucleotide sequence (5′-3′)
1P2	+	1–21	GCTCGAAGTGTGTATGGTGCC
2169P	+	2169–2190	CGTTGCTGGAGATGCTTCGGAC
2463N	−	2482–2463	CGCTTCCACATGACATTGAG
3051N	−	3051–3030	CCCAAGGAAACACAAGCAACAC
4805P	+	4805–4825	AGAGGTAACAGCTGGAGACCG
5198N	−	5217–5198	CTTCCTGATGCTCCACGTAA
7391P	+	7391–7411	TGGTTCTGTGGCAATTGTGTC
7468N	−	7487–7468	AAGTGGAGCGGTACATGATG
8907N	−	8926–8907	CGCTGAGCACGGTTTACTGA
9745P	+	9745–9767	CGTGTGATGGGCTTAGTGTGGTC
9817N	−	9838–9817	AGAAAAGCGCTGCATCAATCAC
10142P	+	10142–10161	GAGTCTTCTAGCTATGCTCC
10763P	+	10763–10782	ACTTTCTACCCATGCCACGC
10952N	−	10952–10933	TGGCCGCAAGCACAAGAAGC
11995N	−	12015–11995	CTAACCCAGATGCTACATACC
12681N	−	12704–12681	GGTTCCTGGGTGGCTAATAACTAC

Nucleotides are numbered according to the published sequence of EAV030 (GenBank ID Y07862) (van Dinten et al., 1997).

Sequence analysis

Nucleotide sequences were assembled and manually edited using CodonCode Aligner v2.0.2 (CodonCode Corporation, Dedham, MA). Sequences were edited with BioEdit v7.0.5.3 (Hall, 1999) and the percentages of the nucleotide identities were determined with EMBOSS Pairwise Alignment Algorithms (http://www.ebi.ac.uk/Tools/emboss/align/). Comparisons of the nucleotide and amino acid sequences were performed with Vector NTI Advance® v11.5.1 (Invitrogen-Life Technologies, Carlsbad, CA).

Phylogenetic analysis

Phylogenetic analysis was conducted using the partial ORF5 sequence of 518 nucleotides (nt numbers 11,296 to 11,813 according to the sequence of the EAV030 strain referenced as Y07862 in GenBank). A total of 388 EAV sequences were used to perform the phylogenetic analysis: 322 from the GenBank database, and 66 additional sequences of partial ORF5 from 26 strains and 40 clones, provided in this study. The GenBank accession numbers of these EAV strains have either been reported previously or are provided in Table 1 (Pronost et al., 2010; Zhang et al., 2007, 2010). The unrooted neighbor-joining tree and dendrogram were constructed using MEGA4.1 and bootstrap analysis was carried out on 1000 replicate data sets (Tamura et al., 2007).

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