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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.

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

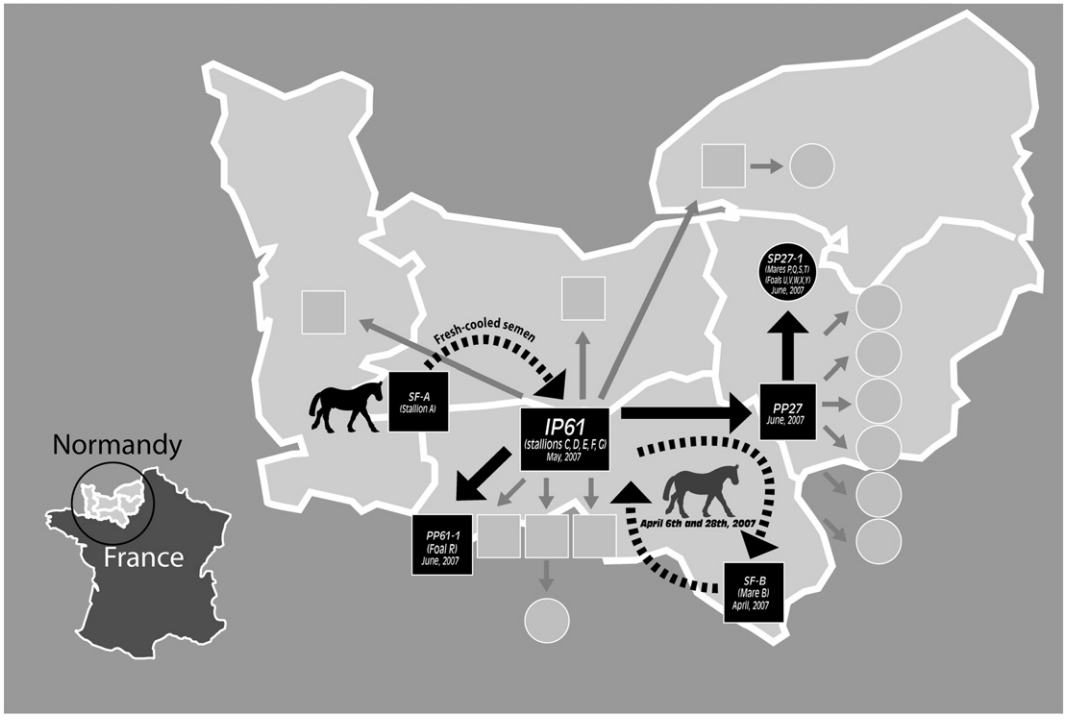

Fig. 1. Schematic depiction of the location of premises to which the EAV infection spreads in Normandy, France. This region is divided in five different areas in Upper (27: Eure, 76: Seine Maritime) and Lower Normandy (14: Calvados, 50: Manche, 61: Orne). Links between the different premises are represented 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.