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Description of the first recorded major occurrence of equine viral arteritis in France

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Keywords: horse; equine arteritis virus; RT-PCR; equine; diagnosis; EAV outbreaks, equine virus arteritis; EAV phylogenetic

Summary

Reasons for performing study: **The vast majority of equine arteritis virus (EAV) infections are inapparent or relatively mild, but may occasionally cause outbreaks of equine viral arteritis. The event observed in France during the summer of 2007 was the most important seen in the country, with mortality and disruption of economic activity.**

Objectives: **To describe the different stages seen during the outbreak and to show how molecular tools were used for both the detection and management of the crisis.**

Methods: **EAV detection was performed by real-time reverse transcription–polymerase chain reaction (RT-PCR) in blood, nasal swabs, semen or organ samples. Characterisation of EAV strains was performed by sequencing the ORF5 fragment.**

Results: **The outbreak affected 18 premises in 5 counties in western France, which represented the index, 8 primary and 9 secondary premises. Artificial insemination in draught horses was responsible for the virus spread. Eight mortality cases were observed, including one fetus, 5 young foals and 2 mature horses. Forty-three individuals had positive results by real-time RT-PCR. The range of measured cycle threshold (Ct) values varied from 19.8 to 40.4 depending on the biological samples. Phylogenetic analysis revealed that the 33 isolated strains all clustered within the EU-2 subgroup.**

Conclusions: **The mortality rate attests to the virulence of the strain involved in this outbreak. Real-time RT-PCR was used for the first time in order to follow-up an epidemic disease in horses.**

Potential relevance: **The early detection of 3 signals with high Ct values attest the importance of taking low signals into account in field conditions.**

Introduction

Equine viral arteritis (EVA) is an equine respiratory and reproductive disease caused by equine arteritis virus (EAV) (Timoney and McCollum 1993). The vast majority of EAV infections are inapparent or ralatively mild, but occasionally they may cause outbreaks of EVA characterised by any combination of influenza-like illness in mature horses, abortion of pregnant more and interstitial pneumonia in very young foals (McCollum *et al*. 1998). Mortality rarely occurs and the clinical signs observed in natural infections may vary considerably among individuals and outbreaks, depending on several factors such as the host, route of infection and viral strain. Fatal infections leading to severe fulminating interstitial pneumonia may be observed in young foals, whereas foals up to a few months of age may develop a rapidly progressive 'pneumo-enteritis' syndrome (Holyoak *et al*. 2008). Temporary subfertility associated with reduced libido could also be observed in stallions. Regardless of the infecting virus strain, the majority of naturally infected horses recover uneventfully from EVA.

EAV is the prototype virus of the *Arteriviridae* family (genus *Arterivirus*; order *Nidovirales*) (Snijder and Meulenberg 1998). It is a single-stranded, positive-sense RNA virus with an approximately 12.7 kb genome, including 9 open reading frames (ORFs) (Snijder and Meulenberg 1998). Genetic variability among EAV strains has been described after sequence analysis of ORFs 2b–7 as well as ORFs 3 and 5 encoding, respectively, the GP3 and GP5, which present the most important variations (Balasuriya *et al*. 2004; Zhang *et al*. 2007). The ORF5 has been the most widely used genome region for phylogenetic studies (Stadejek *et al*. 1999). Recently, studies described a quasi-species organisation presenting a North American clade and a European clade with 2 subgroups, EU-1 and EU-2 (Zhang *et al*. 2007). In contrast, ORFs 1b and 7 encoding, respectively, a replicase and the nucleocapsid protein N, were preferentially used for viral nucleic acid detection by reverse transcriptase–polymerase chain reaction (RT-PCR) (Balasuriya and McLachlan 2007). Because clinical EVA mimics various other infectious and noninfectious diseases of the horse, a presumptive diagnosis of EVA should not be based solely on clinical signs. Differential diagnosis includes other respiratory infections such as equid herpesvirus *types 1* and *4* (EHV-1, EHV-4) and equine influenza virus. Laboratory diagnosis of EVA is currently based on one or more of the following criteria: EAV detected in either blood, nasal swabs, semen or organs; seroconversion or significant (≥4-fold) rise of serum neutralising titre to EAV in paired sera; a positive serum neutralising antibody titre (≥1:4) in a directly

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exposed animal with evidence of spread to other horses on the same premises based on virus detection, seroconversion or a significant rise or decline in antibody titre (Timoney and Creekmore 2006).

Since the recognition of EVA in 1953, outbreaks of the disease have been reported from Europe, Canada and USA (Holyoak *et al*. 2008). Prior to a recent study reporting the genetic variability of 22 French EAV strains by comparative sequence analysis of ORFs 2a–7 (Zhang *et al*. 2007), only few data were published regarding the situation of EVA in France (Moraillon and Moraillon 1978; Stadejek *et al*. 1999; Mittelholzer *et al*. 2006). Serological surveys and, more recently, molecular biological surveys showed that EAV infection occurs in France among *Equidae*. Nevertheless until summer 2007 only few cases were reported, such as the episode of Grosbois in 1986 or Vincennes in 1994 and one isolated case was reported in a retrospective abortion study (Zientara *et al*. 1998; Leon *et al*. 2008). The aim of this paper is to describe the different stages in the development of the 2007 outbreak in France and to show how molecular tools were used for the first time in the detection and management of the crisis.

Materials and methods

Protocols

Collection of samples: Blood, nasal swabs, semen and organ samples from animals were sent to the laboratory mostly in frozen conditions: necropsy was performed in the LERPE centre (Laboratoire d'Etudes et de Recherches en Pathologie Equine, Dozulé France).

Virology and microbiology analysis included in the differential diagnosis: Until EAV could first be identified, different viruses and bacteria were scrutinised on nasal swabs. Herpesvirus *type 1*, *type 2* and *type 4* were investigated by PCR (Leon *et al*. 2008) and influenza virus was detected by the DFA[1] test in accordance with the manufacturer's instructions. Samples were then maintained at -80°C. Presence of bacteria such as *Streptococcus zooepidemicus*, *Rhodococcus equi*, Mycoplasma and anaerobic bacteria were also investigated by culture on specific media for different samples. Concerning abortion, EHV-1 *Chlamydophila*, *Coxiella* and *Leptospira* were investigated on the fetus using PCR (Leon *et al*. 2006; Leon *et al*. 2009) and *Salmonella* by culture.

Virus isolation: Isolation of EAV from the semen and tissue of the aborted fetus was performed in rabbit kidney cells (RK13, ATCC CCL-37) according to a previously described protocol (Timoney 2008).

Serology: The neutralisation test was carried out according to the OIE recommendations (Timoney 2008).

Real-time RT-PCR: Viral RNA was extracted from the different samples containing EAV using the different target-specific kits (QIAamp viral RNA isolation kit[2], RNeasy Mini Kit[2]) according to the manufacturer's instructions. For EAV detection, ORF7 was RT-PCR amplified using a modified protocol previously described (Balasuriya *et al*. 2002). All the amplifications were performed on a SmartCycler II[3] and each run was standardised by the incorporation of a calibrator (Ct = 28.24 ± 0.5). The Ct value is inversely proportional to the virus load as previously described (Balasuriya *et al*. 2002).

For EAV characterisation, ORF5 was RT-PCR amplified according to the protocol adapted from Sekiguchi *et al*. (1995). The reverse primer was M14 5′-GCAGCCAAAAGCACAAAAGC3′ (12124-12105), and a new forward primer MFO5 5′-GTGGCT ATAGTTTATGTTCTTTTACG3′ (11099–11124) was designed using the reference sequence of EAV reference strain Bucyrus (accession number DQ846750, starting at nt 11099–11124) with the program Vector NTI 91[4].

Comparative sequence and phylogenetic analysis: Both sense and antisense strands of the ORF5 PCR products were sequenced (Biofidal[5]) then assembled and manually edited using CodonCode Aligner 2.0.2. Phylogenetic analysis was conducted using a partial ORF sequence of 518 nucleotides in length (11296–11813), which is one of the most variable regions of EAV ORF5. Multiple sequence alignments were performed using BioEdit Sequence Alignment Editor 5.0.9. Phylogenetic analysis and unrooted neighbour-joining tree was constructed using MEGA 4.1.

Nucleotide sequence accession numbers: The nucleotide sequences reported in this study were submitted to GenBank under accession numbers GU189247–GUI89279.

Results

Clinical detection

Index premises (IP61): In this report, a confirmed case of EAV infection was determined as an animal (1) having an epidemiological link validated by the RESPE (French Epidemiological Network of Equine Diseases) to the index premises (IP61), and (2) meeting one or more of the criteria defined by Timoney and Creekmore (2006). On the basis of available data the spread pattern of EAV during the outbreak may be summarised as follows: the virus spread first from the index premises (IP61) in primary premises (PP) and then to secondary premises (SP; Fig 1).

The practitioners observed the first manifestations of the disease at the end of May 2007. First, a stallion on a draught horse stud (IP61) presented with moderate hyperthermia (38.4°C) and both prepuce and limb oedema. One week later, a second stallion from the same stud was examined for hyperthermia (40°C), anorexia, apathy, prepuce, scrotum and limb oedema, conjunctivitis and suborbital oedema and a thin yellow colouration of the gingival mucosa. A diminution of spermatozoid production and motility was also observed in the semen. A few days later, stallions from the next stalls exhibited similar symptoms associated with, sometimes moderate, respiratory clinical signs. Clinical signs persisted for 3–5 days and were attenuated by anti-inflammatory treatment. At this stage, covering was halted and these stallions were isolated. Other horses in the stud then presented with similar symptoms. Cutaneous manifestations with urticaria and oedema appear 1–2 weeks after the first clinical signs.

The first stallion died 10 days after the early symptoms had been detected. The owners did not authorise investigations immediately. Necropsy revealed a myocarditis and bilateral orchitis. Isolation of *Streptococcus zooepidemicus* was observed first and complementary laboratory investigations on 25 June detected EAV in the testis and spleen by RT-PCR. An epidemiological investigation immediately identified a link between several cases, including those described above and some dating back to May 2007.

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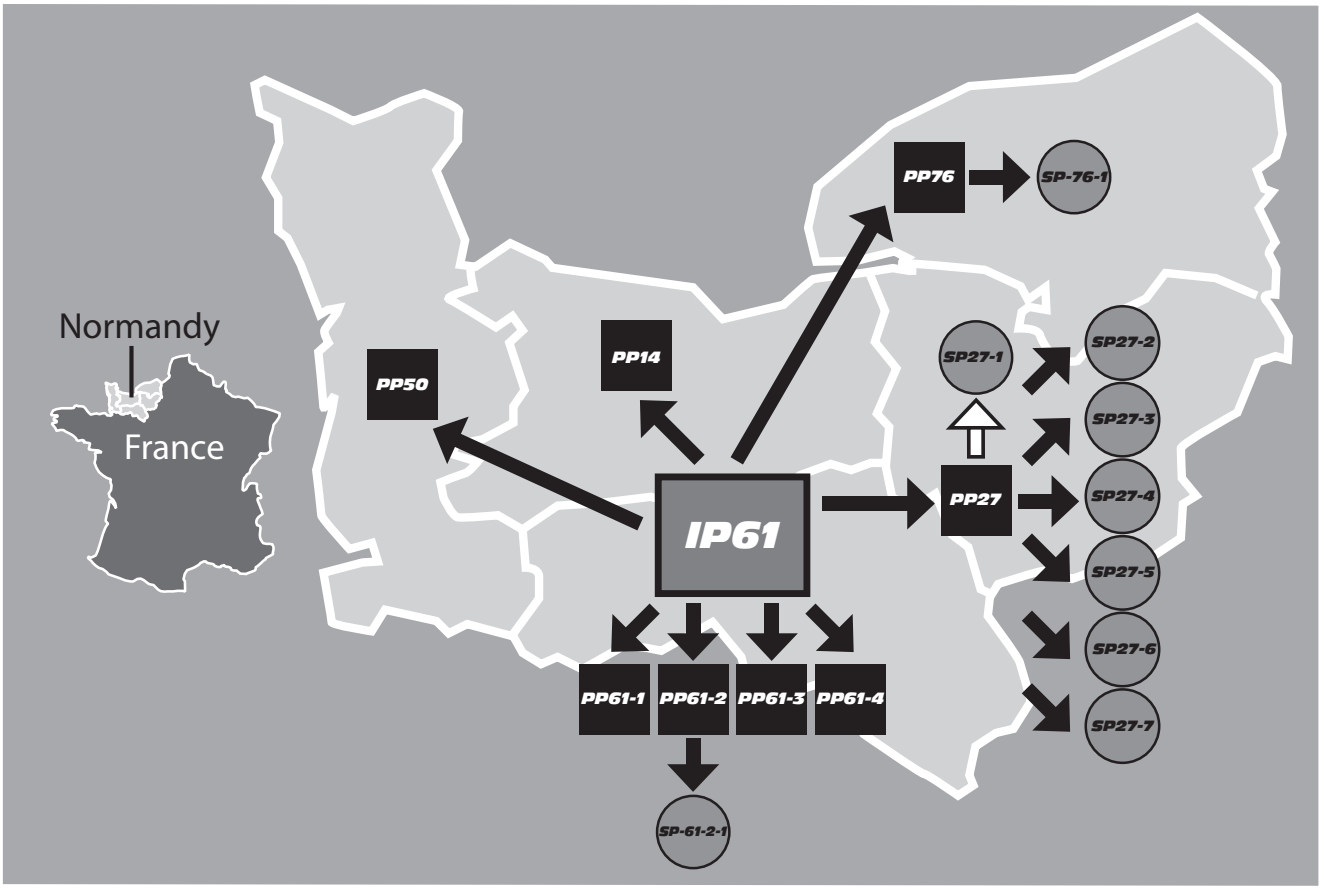

Fig 1: Schematisation of the location of premises to which the infection spread. Grey square: Index premises (IP); black square: primary premises (PP), grey circle: secondary premises (SP). The 2 first numbers correspond to the French areas concerned in the outbreak (61: Orne, 27: Eure, 50: Manche, 61: Orne, 14: Calvados). When several premises exist within the same area they are differentiated by an index number (i.e.: PP61-1 or SP27-1). Black arrow indicates spread by artificially insemination (IA) and white arrow indicates a suspected spread by human vector.

Primary premises (PP) and secondary premises (SP): The outbreak affected 18 premises between the end of May and 2 August (Fig 1). Eight premises involved in the primary spread (PP50, PP14, PP76, PP27 and PP61-1 to PP61-4) were observed in 5 different areas (Manche 50, Calvados 14, Seine Maritime 76, Eure 27 and Orne 61), all in western France. Of these premises, 9 other studs were considered to be involved in the secondary spread (SP27-1 to SP27-7 and SP61-2-1), which was essentially in Eure. Artificial insemination (AI) was responsible for both the spread of the primary and secondary virus except for one stud (SP27-1) where horizontal transmission via humans was suspected. In the absence of any animal or semen link with other studs the practitioner and his tools remained the only potential link with the other infected premises.

The 2 primary premises, PP27 and PP61-1, and the secondary premise, SP27-1, were particularly affected. In PP27 (mating stables), the first symptoms characterised by hyperthermia were observed on 4 June in a mare. Then on 11 June, a second mare was covered 5 days before becoming ill. The third case was observed 1 week later on a saddle horse mare as well as her foal, which also declared the disease 2 days later. One mortality case was reported at this stud on 9 July. Owing to the specific activity of the PP27 stud, several other studs were affected when the mares and their nursing foals returned to their farms (SP27-2–SP27-7).

Concerning stud SP27-1 (a private stud farm with an embryo transplant unit), no movements of horses were noticed to and from the farm during this period. This stud bred young males (1–4 years) for reproduction purposes, as well as 15 mares (11 with nursing foals and 4 donors). The first symptoms were observed in a mare on 29 May and 2 days later, foals also presented with respiratory symptoms. On 8 June, one pregnant mare became ill and recovered before foaling but relapsed 3 days after and was subjected to euthanasia on 20 June due to the uterus prolapse. The necropsy revealed a rupture of the prepubian tendon. Her foal died at 6 days on 24 June. A second foal became ill on 25 June and died a day later.

In PP61-1, death had been previously observed on 15 June but aetiology other than EAV was first suspected. The only confirmed abortion case was noted on 28 June in this stud.

The official end of the outbreak was declared on 26 August 2007 by the 'surveillance committee', which was created just after the alert was given; however, an important monitoring continued until September and new health measures were decided by the expert committee for future mating seasons in several breeds.

Laboratory diagnosis

The first biological evidence concerning the presence of the virus in biological samples was observed on 25 June after amplification by RT-PCR. Regarding respiratory samples, all the other investigations concerning differential diagnoses were negative: absence of influenza virus, EHV-1, EHV-2, EHV-4, *Streptococcus*

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TABLE 1: Value of cycle threshold (Ct) of the qRT-PCR tests of nasal swabs, blood, semen and organs in the 43 positive specimens. Amplification was realised in presence of a standardised RNA control giving a constant Ct value of 28 ± 0.5. F27–F59 represent the isolates from the 33 specimens

Sampling date	Premise§	Source	Specimen	Ct value	Strain code
4 June	IP61	Stallion 1 (10 y)	Testis	34.5	F28
			Spleen	34.2	/
5 June	SP27-1	Mare	Nasal swab	31.9	F29
5 June	SP27-1	Mare	Nasal swab	34.6	F30
15 June	PP61-1	Foal (5 d)	Lung	26.2	F27
			Liver	21.9	/
19 June	SP27-1	Mare	Nasal swab	37.7	/
19 June	SP27-1	Mare	Nasal swab	37.4	/
22 June	SP27-1	Mare (10 y)	Lung	29.7	F31
			Liver	32.2	/
			Kidney	28.1	/
23 June	SP27-2	Mare (19 y)	Blood	36	F32
26 June	SP27-1	Foals (6 d)	Lung	24.6	F33
			Liver	30.8	/
			Kidney	28.1	/
24 June	PP27	Mare	Nasal swab	31.1	/
24 June	PP27	Foal	Nasal swab	28.7	F34
27 June	IP61	Stallion 2	Semen	33.3	F35
27 June	IP61	Stallion 3	Semen	23.8	F36
27 June	IP61	Stallion 4	Semen	32.2	F37
27 June	IP61	Stallion 5	Semen	38.7	/
27 June	IP61	Stallion 6	Semen	19.8	F38
27 June	IP61	Stallion 7	Semen	21.2	F39
27 June	IP61	Stallion 8	Semen	19	F40
27 June	SP27-3	Mare	Nasal swab	27.9	F41
27 June	SP27-1	Foal 4 d	Nasal swab	31.1	F42
28 June	SP27-1	Foal 6 d	Lung	23.9	F43
			Liver	29.7	/
			Kidney	32.6	/
29 June	PP61-1	Fœtus 7 m	Lung	31.2	F44
			Liver	24	/
			Allantochorion	32	/
28 June	IP61	Stallion 9	Semen	29.1	F45
28 June	IP61	Stallion 10	Semen	33.7	F46
29 June	SP27-6	Mare	Blood	36.6	F47
29 June	PP27	Foal	Blood	36.5	F48
			Nasal swab	39.4	/
29 June	PP61-1	Mare	Nasal swab	31.2	F49
2 July	SP27-2	Mare	Nasal swab	40.4	/
2 July	SP27-2	Mare	Nasal swab	29.9	/
2 July	SP27-2	Mare	Nasal swab	35.0	F50
2 July	PP27	Foal	Blood	37.6	F51
2 July	SP27-3	Foal	Nasal swab	31.3	/
2 July	PP76	Mare	Nasal swab	25.6	/
			Blood	34	/
2 July	PP76	Foal (from mare)	Nasal swab	32.3	F52
6 July	SP27-1	Foal	Nasal swab	30.3	/
9 July	SP27-3	Foal (1.5 m)	Lung	28	/
			Liver	35	/
9 July	PP27	Foal	Nasal swab	29.9	F53
9 July	SP27-4	Mare	Nasal swab	35.9	F54
9 July	PP76	Mare	Blood	39.8	F55
10 July	PP27	Foal (1.5 m)	Lung	29.3	F56
			Liver	33.5	/
16 July	SP27-7	Mare	Nasal swab	31.4	F57
25 July	IP61	Stallion 11	Semen	22.2	F58
2 August	IP61	Stallion 12	Semen	31.2	F59

§IP: Index premises; PP: primary premise, SP: secondary premise. The first 2 numbers correspond to the 5 French areas concerned by the outbreak (61: Orne, 27: Eure, 50: Manche, 61: Orne, 14: Calvados).

zooepidemicus, *Rhodococcus equi*, Mycoplasma and anaerobic bacteria. For EAV, 3 signals (2 from nasal swabs, SP27-1; one from testis, IP61) were concomitantly observed in the same run. The 3 Ct values (38–42, data not shown) were all characterised by very weak signals and would need confirmation even if all the controls were confirmed. The 2 nasal swabs and the testis were confirmed positive following a new amplification out of a new extraction with Ct comprised between 31.9 and 34.6 (Table 1). A day later, samples of lung and liver from a very young foal (5 days old) were also positive with low Ct values (respectively 26.2 and 21.9) suggesting a high viral load. This was confirmed by virus isolation in cell culture after only 2 days. Histopathological analysis revealed a necrotising arteritis in both liver and lungs, as well as an interstitial inflammation in lungs and kidneys.

Considering this first established case of mortality in a very young foal (F27), all the cases of abortion and foal mortality sent to

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the laboratory between May and 15 June were tested for EAV and were all negative (data not shown).

Table 1 illustrates the diversity of the different specimens that gave positive results by RT-PCR during this crisis: 58 samples (20 nasal swabs, 21 organs, 11 semen and 6 blood samples) collected from 43 individuals (1 fetus, 13 foals, 17 mares and 12 stallions, essentially draught and saddle horses) between 5 June and 2 August 2007. The range of Ct values were, respectively, 25.6–40.4 for nasal swabs, 34–39.8 for blood, 19.8–38.7 for semen and 21.9–35 for organs (all values are summarised in Table 1). Seventy-four percent (32/43 cases) presented a Ct value <35 and of the 26% with a Ct >35, 40% (4/10) could not be isolated by cell culture. All the blood samples analysed presented a high Ct value attested from a low viral load. Low Ct values attested from a high viral load are observed in tissue of the fetus and very young foals, in particular in the lung and liver.

Concerning EAV persistently infected stallions, 5 became negative in September although 6 were castrated in October. Overall, seroconversions were detected in 21 animals. Evidence of seroconversion was sometimes impossible to assert in the absence of an initial serological status. During the outbreak, several samples from other areas (east and south of France) were sent to the laboratory for EAV testing in horses presenting symptoms suggestive of EVA; however, no positive samples were detected in these areas.

Phylogenetic analysis

The majority of ORF5 sequences deposited in GenBank are partial ORF5 sequences (positions 11296–11813). This sequence region of the 33 EAV isolates from the 2007 Normandy outbreak analysed in this study and 238 previously published EAV strains isolated around the world (total 271) were used for phylogenetic analysis. The GenBank accession numbers and references are given in Zhang *et al*. (2007) for the 238 previously published EAV strains and in this paper for the 33 EAV strains involved in this study. ORF5-based phylogenetic analysis clustered the globally isolated strains of EAV into distinct North American and European groups; the latter could be divided into European subgroup 1 and European subgroup 2 (Fig 2). In the case of the 33 EAV strains isolated during the outbreak (F27–F59), all clustered with the EU-2 subgroup and all were grouped in the FR2007 cluster except F38, which is more closely related to strains 6, 11, 154 and 155. Strains 6 and 11 were 2 strains isolated from French Thoroughbreds, respectively, in 2001 and 2003 and strains 154 and 155 are 2 strains isolated from German Thoroughbreds in 1994 and 1995. All the strains presented between 99.6 and 100% homology with F27, the first strain sequencing during the crisis, except F38, which differed 6% (32/518) in the nucleotide sequence.

Discussion

During this outbreak in France occurring in spring and summer 2007, there was evidence of a spread of infection from the index premises to 17 premises; 16 of which semen had been sent off and in one (SP27-1) where horizontal transmission by humans was suspected. Regarding mortality, this last stud was the most affected by the virus. In the other studs, spread could also not be excluded after an infected mare had been returned to the stud. The presence of EAV in nasal swabs is clearly suggestive of the respiratory features of the disease as reported in USA in 1978 and 1993 when the 2 occurrences led to dissemination of the virus among a significant number of states (McCollum and Swerczek 1978; Dwyer *et al*. 1993). The crisis described here is more similar to the recent outbreaks (Wood *et al*. 1995; Powell and Timoney 2006) associated with semen transfer. The virus has again been proven to be readily transmitted by either fresh-cooled or frozen semen (Metcalf 2001; Powell and Timoney 2006). Horses affected with EVA exhibited in this outbreak, to a greater or lesser degree, many to all of the clinical signs previously reported (Timoney and McCollum 1993). No evidence of infection was detected in the yearlings on any affected premises. Seven mortality cases (1 mature horse, 5 foals and 1 fetus) were demonstrated, even if the necropsy of the stallion concluded a myocarditis as the probable cause of death. One mare that had a rupture of the prebubian tendon was subjected to euthanasia and was also infected by EAV. The fetus was aborted during the 7th month of gestation, as already reported in other studies (Timoney and Creekmore 2006). Regarding the mortality cases, this outbreak is one of the most important observed crises. The involvement of young foals attested the virulence of the strain involved in this outbreak. Moreover, other young animals were strongly suspected (even without laboratory analysis) to die after contamination by this strain during the transition period between observation of the first clinical signs and the first confirmation by RT-PCR. The first alert was given on 25 June 2007 after confirmation of EAV by RT-PCR. As observed during this event, there is a non reducible period between the starting point of the clinical signs in the index case and the laboratory confirmation. This duration varies for the different events but each time several weeks were needed during the total crisis period of 2–4 months (McCollum and Swerczek 1978; Timoney 1984; Dwyer *et al*. 1993; Wood *et al*. 1995; McCollum *et al*. 1998; Powell and Timoney 2006). The 2 major reasons for this delay are the lack of specificity of primary clinical signs, which could be amplified in a country not familiar with EVA, and the fact that the diagnosis unit in laboratories first look for the pathogens requested by practitioners. This was also reinforced by the race concern. Indeed, surveillance and more specifically EVA symptomatology associated with this infection was at this time less important among draught horse owners and farm managers than in other breeds such as Standardbreds or Thoroughbreds. This delay could also explain why the virus was detected concomitantly in the index premises (IP61) and in secondary premises (SP27-a).

This sequence of events emphasised the critical step of diagnosis, which was the event leading to the declaration of the disease and the organisation of national surveillance. During this crisis, the presence of the virus was first confirmed by RT-PCR from 2 nasal swabs and organs, respectively, from 2 mares and one stallion in 2 areas and 2 days later from the culture and histopathology results obtained from a young foal. This outbreak demonstrated the paramount importance of taking into account low signal (even over a Ct value >40) when the characteristic amplification curve was present. Even if it is well established that Ct value is not a usual unit for viral quantification, this still remains informative, associated with the aspect of the curve in a qualitative analysis. Furthermore, defining an arbitrary cut-off is not ideal (Burns and Valdivia 2008). During laboratory analysis, working on 'field samples' necessitated retesting frozen nucleic acid after a primary first investigation for other respiratory viruses and this could lead to loss of some signal compared to experimental procedures. This could be due to the conservation step of the sample and the possible diminution of the PCR efficiency observed

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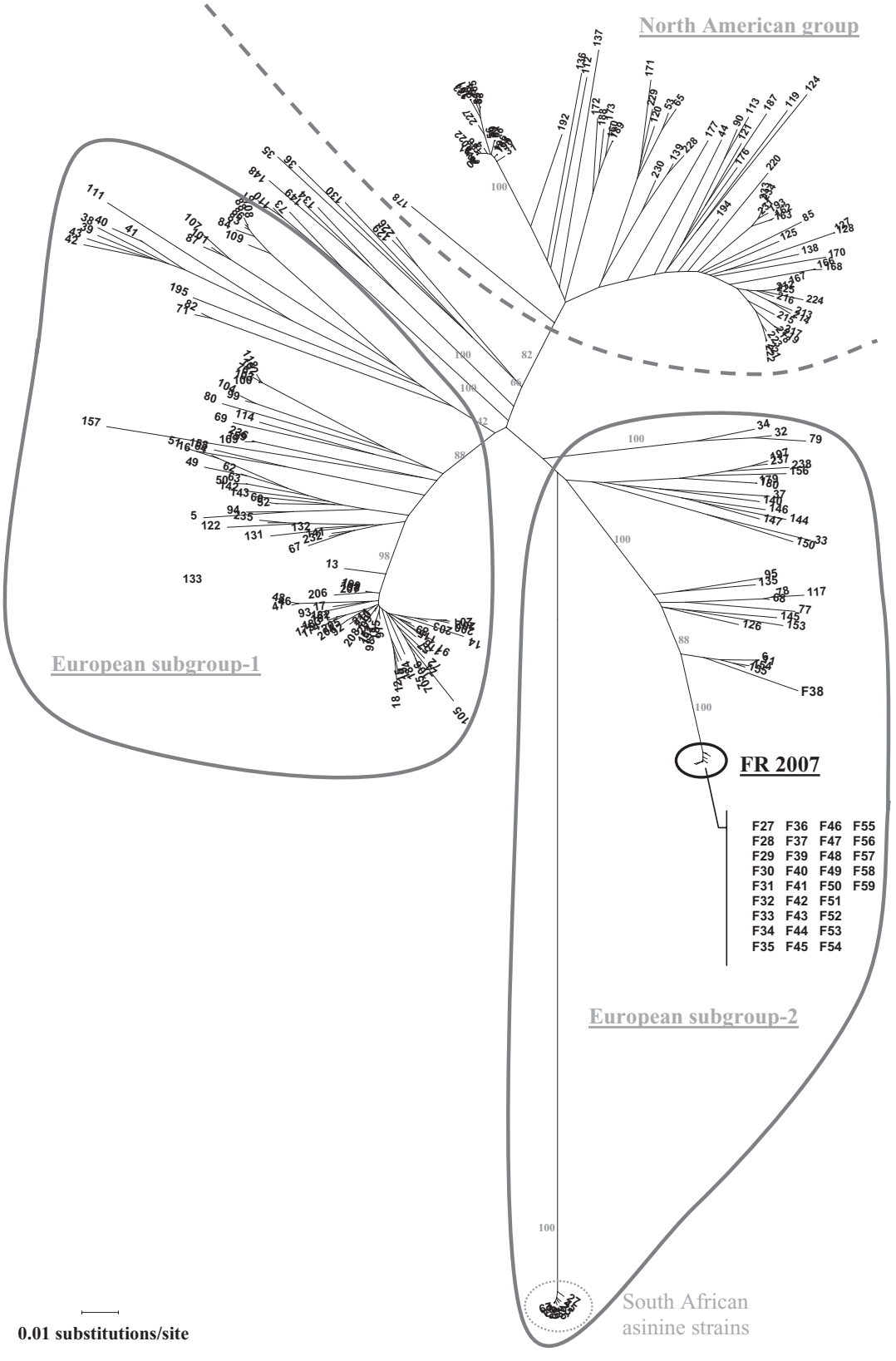

Fig 2: Phylogenetic analysis of the ORF5 of 271 EAV strains: The partial ORF5 nucleotide sequences (518 nucleotides in length from positions 11296 to11813) of 33 French EAV strains isolated during the 2007 Normandy outbreak and 238 previously published EAV strains were used for the phylogenetic analysis. Multiple sequence alignments were carried out by using ClustalX 1.83 and the neighbour-joining tree is shown here. The boostrap analysis was carried out on 1000 replicate data sets and values are indicated adjacent to the major branching point. Strain numbers correspond to the sixth column of Table 1. Strains that were overlapping or closely adjacent to each other on the tree are boxed and shown together. The North American and European groups are separated by a dotted line. European subgroup 1 and European subgroup 2 are indicated. The 33 EAV isolates from the French outbreak analysed in this study are indicated in bold and grouped in the cluster FR2007 excluding F38.

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between field samples and the constructed standard. The design of the PCR test, the amplification steps varying from one laboratory to another, the control of the apparatus being used, the sample itself as well as the experience of the technicians are all essential for arriving at a good diagnosis. This is, therefore, another argument for promoting both harmonisation and standardisation of the procedures among laboratories (Lu *et al*. 2008). Virus isolation still remains the 'gold standard method' because studies performed on semen and tissue culture fluid demonstrated in a few cases the lack of RT-PCR sensitivity concerning samples with a low viral load (Lu *et al*. 2008). During this outbreak, we nevertheless observed that 4 samples were positive by RT-PCR and negative by culture isolation after 4 passages. Each time these discrepancies were observed for samples presenting a low viral load attested by a high Ct value (>37), which could be explained by the conservation of the biological sample.

Another advantage of the RT-PCR during this crisis was the rapidity in obtaining results. The characteristic of the thermocyclers allowed each sample to be processed in the laboratory within 2 h. This was important to allow RESPE to disseminate in real time the evolution of the crisis after it had been declared and to reassure owners when samples were negative. To our knowledge, it was one of the first outbreaks that used molecular tools in equine species, even 2 months before the influenza outbreak in Australia (Foord *et al*. 2009).

The strain (F27) from the first established EVA case was sequenced 2 days after detecting the first signal. This did not give information about the pathogenicity, but nevertheless attested the presence of a new strain. The creation of a cluster (FR2007) after sequencing the different strains involved during this event is characteristic of an outbreak. The origin of the crisis still remains unknown. However, the observation of this cluster suggests that a single origin could be suspected even if one strain (F38) was outside the cluster and was more closely related to 4 other strains (154, 155, 6 and 15), respectively, from Germany, Poland and France for the last 2. Considering the unknown status of stallion 6 (F38) before the crisis and the identified stability of the strain during the crisis, it could be suggested that this stallion was an asymptomatic carrier. Different hypotheses may be proposed for the origin of the crisis: (1) an infected animal could be the source of the spread by the respiratory route after contact with horses from the IP61, and (2) a stallion could be the source of the infection by evolution of a variant, which could have became more pathogenic. These different hypotheses should be investigated to determine the origin of the crisis as previously described (Balasuriya *et al*. 1999).

Although most EAV strains are of low virulence, outbreaks of EVA with severe clinical symptoms still occur as attested by this event. Although this first major French outbreak was moderate, it is a reminder of the potential risk as well as the necessity of a close collaboration between owners, practitioners, laboratories and administration to efficiently fight against the virus.

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Manufacturers' addresses

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[3]Cepheid, Maurens-Scopont, France.
[4]Invitrogen, Cergy Pontoise, France.
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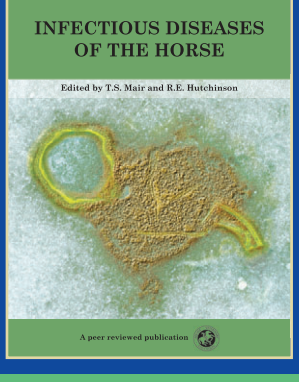

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