Genetic stability of equine arteritis virus during horizontal and vertical transmission in an outbreak of equine viral arteritis

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An imported carrier stallion (A) from Europe was implicated in causing an extensive outbreak of equine viral arteritis (EVA) on a Warmblood breeding farm in Pennsylvania, USA. Strains of equine arteritis virus (EAV) present in the semen of two carrier stallions (A and G) on the farm were compared to those in tissues of foals born during the outbreak, as well as viruses present in the semen of two other stallions that became persistently infected carriers of EAV following infection during the outbreak. The 2822 bp segment encompassing ORFs 2–7 (nt 9807–12628; which encode the Gs, GP3, GP4, G1, M and N proteins, respectively) was directly amplified by RT–PCR from semen samples and foal tissues. Nucleotide and phylogenetic analyses confirmed that virus present in the semen of stallion A initiated the outbreak. The genomes of viruses present in most foal tissues (10/11) and serum from an acutely infected mare collected during the outbreak were identical to that of virus present in the lung of the first foal that died of EVA. Virus in the placenta of one foal differed by one nucleotide (99.9% identity) from the predominant outbreak virus. The relative genetic stability of viruses that circulated during the outbreak contrasts markedly with the heterogeneous virus populations variously present in the semen of persistently infected stallions on the farm. These findings are consistent with the hypothesis that the carrier stallion can be a source of genetic diversity of EAV, and that outbreaks of EVA can be initiated by the horizontal aerosol transmission of specific viral variants that occur in the semen of particular carrier stallions.

Introduction

Equine arteritis virus (EAV) is a member of the genus Arterivirus, family Arteriviridae in the order Nidovirales (Cavanagh, 1997), and is the causative agent of equine viral arteritis (EVA) of horses (Doll et al., 1957a). Outbreaks of EVA are characterized by any combination of systemic illness of adult horses, abortion of pregnant mares, interstitial pneumonia of young foals and persistent infection of stallions (Doll et al., 1957a, b; Golnik et al., 1981; Timoney et al., 1986, 1987, 1992; Carman et al., 1988; Vaala et al., 1992; Del Piero et al., 1995, 1997). EAV is horizontally transmitted either by aerosol during outbreaks of EVA or veneerally via the breeding of an infected stallion to susceptible mares, and vertically through congenital infection of foals born to mares infected late in gestation (Timoney et al., 1987, 1992; Vaala et al., 1992; Timoney & McCollum, 1993; Glaser et al., 1996). Dissemination of EAV by fomites such as vehicles, twitches, artificial vaginas and shanks can be an important source of infection in some outbreaks (Collins et al., 1987; Timoney & McCollum, 1988, 1993). The persistently infected carrier stallion clearly plays an important role in perpetuation and sexual dissemination of EAV. The persistence of EAV in the male reproductive tract is testosterone-dependent (Timoney & McCollum, 1993). We recently have shown that EAV behaves as a quasispecies during persistent infection of carrier stallions, with regular emergence of novel genotypic and phenotypic viral variants (Hedges et al., 1999).

The EAV genome is a linear 12.7 kb, positive-sense, polyadenylated, single-stranded RNA molecule that contains eight ORFs (den Boon et al., 1991; de Vries et al., 1997). ORFs 1a and 1b encode the viral replicase and are located at the 5’
end of the genome. Four ORFs (2, 5, 6 and 7) that encode the known structural proteins of EAV, and two ORFs (3 and 4) that encode two glycoproteins of unknown function, are located downstream of ORF1b at the 3’ end of the genome (de Vries et al., 1992). ORFs 5 and 6, respectively, encode the major envelope glycoprotein G\textsubscript{L} (30–42 kDa) and the non-glycosylated envelope protein M (17 kDa). ORF2 encodes the minor envelope glycoprotein G\textsubscript{s} (25 kDa) and ORF7 encodes the phosphorylated N protein (14 kDa) that forms the icosahedral nucleocapsid core that encapsidates genomic RNA.

The circumstances and clinical features of an extensive outbreak of EVA that occurred on a Warmblood breeding farm in Pennsylvania, USA, during the spring of 1996 have been recently reported (McCollum et al., 1999). This outbreak provided an unparalleled opportunity to investigate the evolution of EAV during an outbreak of EVA and during persistent infection of stallions that became carriers of EAV following the outbreak. The objectives of the present study were to confirm the source of the outbreak and to determine the degree of virus genetic heterogeneity that was generated during the outbreak.

Methods

**Description of the outbreak.** Circumstantial evidence suggested that the EVA outbreak was initiated by one of two known EAV carrier stallions (A and G) that were imported to the farm from Europe in 1995 (McCollum et al., 1999). One of the stallions (A) was known to be a carrier prior to export. Semen was initially collected from stallion A on March 13 and again on March 18, 1996. On March 22, the first evidence of clinical EVA was detected in another stallion (M) that was stabled in the same barn as stallion A. The infection rapidly spread to other stallions as well as to a group of mares and foals on the farm. All clinically unaffected horses were vaccinated with the modified live virus (MLV) vaccine ARVAC on either April 1 or April 4, 1996. The EVA outbreak lasted several weeks and clinical signs were observed in a total of 23 horses: four stallions, seven mares and 12 foals (McCollum et al., 1999). One mare aborted at 10 months of gestation and three foals died (two with interstitial pneumonia) during the outbreak. Two stallions infected during the outbreak subsequently became shedders of EAV (P and R; Table 1).

**Isolation of viral RNA, RT–PCR amplification, molecular cloning and sequencing.** Viral RNA was directly isolated using the QiAmp Viral RNA isolation kit (Qiagen) from homogenates of placenta from one aborted foetus and six congenitally infected neonatal foals, and from tissues of two foals that died during the outbreak (Table 2). Viral RNA was also isolated from the serum of an acutely infected mare (BN), from the semen of two stallions (P and R) that became persistently infected carriers following the outbreak, and from the semen of two known carrier stallions (A and G) that were present on the farm prior to the outbreak (Table 1). ORFs 2–7 and flanking portions of the EAV genome (2923 bp) were amplified by RT–PCR using RT–PCR using RT enzymes (5\textsubscript{th} Turbo DNA polymerase (Stratagene) enzymes, as previously described (Hedges et al., 1999). Twenty PCR reactions (50 µl per reaction) derived from each sample were pooled and gel-purified and both sense and non-sense strands were sequenced with the PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) primed by internal sequence-specific primers (Hedges et al., 1996, 1999). The PCR amplicons from three semen samples (A2, P2 and R2) and three placenta (BT, HV and RC) were cloned using the pT7Blu-3 Perfectly Blunt cloning kit (Novagen), as previously described, and ORF5 of each clone was then sequenced (Hedges et al., 1999). The estimated rate of misincorporations in RT–PCR and cloning was 1 artifactual substitution per 1667 bp (based on the error rate of retrovirus RT enzymes (5.5 × 10\textsuperscript{−4}) and Pfu Turbo DNA polymerase (1.5 × 10\textsuperscript{−6} substitutions/bp/cycle)).

**Sequence and phylogenetic analysis.** Sequence data were analysed with the Sequencher 3.0 (Gene Codes) and HIBIO MacDNASIS pro version 3.5 (Hitachi) software programs using a Macintosh Power PC. Sequences were aligned using the TOPIR program of the Wisconsin package (CCG Version 8.0 software; Genetic Computer Group) and CLUSTAL W version 1.7 (Thompson et al., 1994). Genetic distances between each pair of sequences were calculated using the DNADIST program of the Phylogenetic Inference Package (PHYLIP; Felsenstein, 1985, 1993) version 3.5c based on the Kimura two-parameter model (Kimura, 1980; Felsenstein, 1993) with a transition/transversion ratio of 2. The distance matrices generated from DNADIST were utilized in the FITCH program (least-squares method) to generate phylograms (Fitch & Margoliash, 1967). Bootstrap resampling and tree inference were performed using the SEQBOOT, DNADIST, FITCH and CONSENSE programs from PHYLIP (Felsenstein, 1985). Phylogenetic relationships among the clones were also estimated by the neighbour-joining (NJ) method (Saitou & Nei, 1987). Branch lengths for the NJ tree were estimated by the maximum-likelihood method (transition/transversion ratio of 2, gamma shape parameter estimated by parsimony of 0.602664, proportion of invariable sites estimated by program) using PAUP* beta test version 4.0b1 (Swofford, 1997). A bootstrap value > 70% was interpreted as reliable support for groups (Hillis & Bull, 1993).  

Results

**Sequence and phylogenetic analyses and source of the outbreak**

The sequences of EAV strains present in the semen of two
Table 2. Isolation of EAV from foetal tissues collected during the EVA outbreak

<table>
<thead>
<tr>
<th>Foal ID</th>
<th>DOB*</th>
<th>Outcome</th>
<th>Tissue†</th>
<th>EAV titre (p.f.u./g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT</td>
<td>21 March 1996</td>
<td>Pneumonia; died 27 March 1996</td>
<td>Lung#</td>
<td>&gt; 10^4</td>
</tr>
<tr>
<td>ST</td>
<td>25 March 1996</td>
<td>Died 30 March 1996</td>
<td>Spleen</td>
<td>1.25 × 10^4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lung</td>
<td>1.3 × 10^4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lymph node</td>
<td>4 × 10^3</td>
</tr>
<tr>
<td>HR</td>
<td>1 April 1996</td>
<td>Sick; recovered</td>
<td>Placenta</td>
<td>3.5 × 10^3</td>
</tr>
<tr>
<td>JD§</td>
<td>10 April 1996</td>
<td>Sick; recovered</td>
<td>Placenta</td>
<td>10^5</td>
</tr>
<tr>
<td>DR§</td>
<td>13 April 1996</td>
<td>Pneumonia; died 17 April 1996</td>
<td>Placenta</td>
<td>2 × 10^5</td>
</tr>
<tr>
<td>RO§</td>
<td>13 April 1996</td>
<td>Weak foal; recovered</td>
<td>Placenta</td>
<td>10^5</td>
</tr>
<tr>
<td>HV§</td>
<td></td>
<td>Aborted 13 April 1996</td>
<td>Placenta</td>
<td>6 × 10^4</td>
</tr>
<tr>
<td>AP§</td>
<td>15 April 1996</td>
<td>Normal</td>
<td>Placenta</td>
<td>1.02 × 10^4</td>
</tr>
<tr>
<td>RC§</td>
<td>19 April 1996</td>
<td>Normal</td>
<td>Placenta</td>
<td>3 × 10^3</td>
</tr>
</tbody>
</table>

* DOB, Date of birth.
† Tissues that were selected for direct RT–PCR sequencing of ORFs 2–7.
‡ ORF5 was also sequenced from cloned PCR amplicons from these viruses.
§ Dam vaccinated against EVA 4/1/96 (ARVAC).

stallions (A and G) that were imported to the farm from Europe were compared to those of archived strains of EAV isolated from previous outbreaks of EVA and from the semen of other carrier stallions, and to viruses present in a variety of tissues from foals and serum from a mare collected during the outbreak, and in semen samples collected from two stallions that became carriers following the outbreak. Viruses from stallions A and G differed from the North American Bucyrus strain of EAV by 290–298 nt [87.6–87.9% identity (A1–A4)] and 286–294 nt [87.7–88% (G1–G4)], respectively (Doll et al., 1957; den Boon, et al., 1991), and differed from each other by 52 nt (98.2% identity) immediately following importation (A1 and G1 samples; Table 1). Two years later, the master sequence of viruses present in the semen of the two stallions (A4 and G2 samples) differed by 61 nt (nine additional nucleotide differences), indicating that one or both of the viruses had evolved during persistent infection. Comparative nucleotide analysis of direct RT–PCR products encompassing ORFs 2–7 (2822 bp; data not shown) as well as phylogenetic analysis of ORF5, indicated that the strains of EAV present in the semen of the two imported persistently infected stallions (A and G) were most closely related to EAV strains that originated in Europe (Fig. 1a). These viruses (A and G) shared more recent common ancestry with a virus contained in semen imported from Europe that caused abortion of a mare in Washington State in 1997 (WA97; Balasuriya et al., 1998) than with other European-derived EAV strains (Fig. 1a). This result was very strongly supported by bootstrapping.

Phylogenetic analysis of ORFs 2–7 of the master sequences of viruses that were present in foal tissues and sequential semen samples from the four persistently infected stallions was consistent with the hypothesis that stallion A precipitated the outbreak (Fig. 1b). The various viruses evaluated from the farm segregated into two main lineages. One monophyletic group included all viruses from the outbreak, the various semen viruses from the two stallions (P and R) that became persistently infected with EAV following the outbreak, and the viruses present sequentially in the semen of stallion A. The virus that was present in the semen of stallion R (R1) during the outbreak was identical to the viruses present in the foal tissues, and these viruses cluster together to form a clade. The second monophyletic group includes viruses sequentially present in the semen of stallion G, which was not involved in the outbreak, and the MLV vaccine strain of EAV.

The virus in the lung of the first foal that died during the outbreak (BT lung) differed by 14 nt (99.5% identity) from the virus in the semen sample (A2) that was collected from stallion A 4 days prior to the outbreak (Fig. 2). With the exception of one virus (RQ), the other viruses in foal tissues collected during the outbreak, in serum from an acutely infected mare (BN) and from semen of an acutely infected stallion (R1) were identical. Virus present in the placenta of foal RQ differed from the other outbreak viruses by only one nucleotide (99.9%). Thus, there were no obvious sequence differences between viruses present in the tissues of the aborted foal, the foals that developed pneumonia or systemic illness, and in the placentas of those foals that exhibited no clinical evidence of EAV infection. The viral sequences from the semen of the two stallions (P2 and R2) that became persistently infected after the outbreak differed by 44 nt (98.4% identity) after 12 months of persistent infection, indicating that each virus evolved independently during the course of persistent infection (Fig. 1b).

The MLV vaccine virus (ARVAC) that was used to immunize clinically unaffected horses on the farm during the
Fig. 1. (a) Phylogram (FITCH) derived from ORF5 nucleotide sequences of EAV strains of North American and European origin and the MLV vaccine strain (ARVAC). Stallions A and G [A2 and G1 viruses (boxed) are included as representative samples] were carriers of EAV prior to the outbreak. All the other sequences used in this analysis, other than CA97 which was isolated from an imported carrier stallion in California in 1997, have been previously reported (Balasuriya et al., 1995, 1998).

(b) Phylogram (FITCH) derived from sequences of ORFs 2–7 of viruses present in the semen of carrier stallions (A, G, P and R).
The genetic stability of EAV during horizontal and vertical transmission (Fig. 3). A total of 78 clones that included the entire ORF5 was analysed, including 14–20 clones from each semen sample and 28 clones from the various foal tissues. With the exception of one clone from stallion A (semen sample A2), sequences of the clones from the semen of the three stallions and the foal tissues segregated into two main clades (Fig. 3). All clones from the semen of stallion R and the foal tissues grouped with a single clone from the semen of stallion A (A07) to form one clade, indicating that transfer of a viral variant in the semen of stallion A probably initiated the outbreak. Within this clade, clones from the semen of stallion R formed a monophyletic group that was reliably supported by bootstrapping. The majority of the clones from stallion A (14/16) and all of the clones from stallion P (P2) formed the second clade. All clones (14/14) from semen sample P2 and two clones from A2 (A02 and A23) formed a reliably supported monophyletic group within this clade, suggesting that stallion P might have been originally infected with a variant present in the A2 semen that was different from the variant that infected all other animals in the outbreak.

Analysis of clone sequences from the outbreak and three carrier stallions

ORF5 sequences of clones derived from the RT–PCR products directly amplified from the semen of carrier stallions A, P and R (samples A2, P2 and R2), and tissues from three foals (BT, HV and RC) were compared to further characterize the genetic variation of EAV during horizontal and vertical transmission (Fig. 3). A total of 78 clones that included the entire ORF5 was analysed, including 14–20 clones from each semen sample and 28 clones from the various foal tissues. With the exception of one clone from stallion A (semen sample A2), sequences of the clones from the semen of the three stallions and the foal tissues segregated into two main clades (Fig. 3). All clones from the semen of stallion R and the foal tissues grouped with a single clone from the semen of stallion A (A07) to form one clade, indicating that transfer of a viral variant in the semen of stallion A probably initiated the outbreak. Within this clade, clones from the semen of stallion R formed a monophyletic group that was reliably supported by bootstrapping. The majority of the clones from stallion A (14/16) and all of the clones from stallion P (P2) formed the second clade. All clones (14/14) from semen sample P2 and two clones from A2 (A02 and A23) formed a reliably supported monophyletic group within this clade, suggesting that stallion P might have been originally infected with a variant present in the A2 semen that was different from the variant that infected all other animals in the outbreak.

Fig. 2. Number of pairwise nucleotide differences and percentage differences between virus sequences (ORFs 2–7) amplified from semen samples and foal tissues before, during and after the outbreak. All virus sequences were compared to the virus present in the semen of stallion A immediately prior to the outbreak (A2). Viruses are identified as described in Tables 1 and 2.
In contrast to the heterogeneous (quasispecies) virus population in the semen of carrier stallions, the clones and direct RT–PCR products from the various foal tissues evaluated from the outbreak had relatively uniform sequences. Conversely, there were several deletion mutants in the clones from stallion P (3/14 P2 clones: nt 11419, 11578–11579 and 11234–11279, respectively), one in the clones from stallion R (1/20 R2 clones; nt 11452), and none in the clones from stallion A. Sequence analysis of clones from the P2 and R2 semen samples from stallions P and R that became persistently infected after the outbreak indicate that genetic diversity was generated during the course of persistent EAV infection in these carrier stallions.

**Comparative amino acid sequence analysis of Gs, GP3, GP4, G1, M and N proteins**

The nucleotide sequences of each ORF (2–7) of the various viruses present during and following the outbreak were translated into amino acid sequences (Fig. 4). The individual viral proteins of the outbreak viruses were compared to those of the virus present in the semen of stallion A at the time of the outbreak (A2). Most of the viruses had one or two amino acid
changes in four proteins (GS, GP3, GP4, M and N) compared to A2, whereas the N and GP3 proteins were conserved during the outbreak. The GP4 and M proteins of the virus present in the placenta of foal RQ had unique changes at amino acid positions 37 and 114, respectively. There was a single amino acid change (K to N) at position 61 of the GL protein of all the outbreak viruses and two of the semen viruses (R1 and R2). In contrast, the amino acid at position 61 of the P1 and P2 viruses was maintained in its original status (K). This site has previously been identified as being important for virus neutralization (Balasuriya et al., 1997).

We also examined amino acid substitutions in the region encompassing amino acids 51–120 of the predicted GL protein of the various clones. This region includes the V1 variable region of the GL protein [which contains three of the four major neutralization sites (B, C and D) of EAV] and the N-terminal 10 amino acids flanking the V1 region (Balasuriya et al., 1997). Alignment of deduced amino acid sequences of the clones derived from the semen samples and foal tissues showed that there are significantly more amino acid substitutions in the area of amino acids 58–84 of the semen viruses. The majority of these substitutions were in the region of neutralization sites B and C (Fig. 5). The stability of this region during the outbreak (conserved in 22/28 clones) contrasts with its variation in the persistently infected stallions (conserved in 7/16, 10/14 and 12/20 clones from stallions A, P and R, respectively). Some non-synonymous substitutions and deletions in clones derived from semen viruses created new termination codons, thus encoding truncated proteins, whereas others created modified proteins through the introduction of different amino acids. One of the clones from the foal tissues did acquire a third putative glycosylation site at amino acid position 60 (clone derived from RC), but variation was more marked in the clones derived from semen, including loss of the primary glycosylation site at position 56 of one clone from stallion R2.

Amino acid changes in the GS, GP4 proteins also led to the loss of two putative glycosylation sites (at positions 28 and 29) in all semen viruses (P1 and R1; Fig. 4). Both glycosylation sites reappeared in the virus present in the semen of stallion P after one year, whereas only the GP4 site reappeared after one year in the virus present in stallion R. The GS, M and N proteins of the viruses from the two imported European stallions (A and G) were identical to each other, whereas the GP3, GP4 and GL proteins differed significantly (Fig. 4). A potentially significant finding was the loss of two putative glycosylation sites at positions 28 and 29 of the GP3 protein of the G2, G3 and G4 viruses during persistent infection in stallion G.
**Discussion**

Sequence and phylogenetic analyses were used to determine the origin and molecular epidemiological features of an outbreak of EVA that occurred on a Warmblood breeding farm in Pennsylvania. The viruses present in the semen of stallions A and G that were imported from Europe were very similar to other European strains of EAV, and viruses in the tissues of foals born during the outbreak were very similar to those in the semen of stallion A. Transmission of a variant(s) selected from the EAV quasispecies population present in the semen of the stallion A clearly was responsible for the outbreak, and the EAV population that circulated during the outbreak was genetically stable during repeated horizontal and vertical passage in horses.

Previous sequence analyses indicated that EAV behaves as a quasispecies and that genetic and phenotypic divergence can occur during persistent EAV infection of carrier stallions (Hedges et al., 1999). Major EVA outbreaks are the result of periodic emergence of novel genetic and phenotypic variants of the virus. However, the mechanisms involved in selection and emergence of virulent viral variants remain unclear. Selection of such variants might occur either during initial virus transmission or at some time thereafter. Data from this study indicate that selective amplification and transmission of a viral variant present in the semen of carrier stallion A was the source of this outbreak of EVA. This viral variant apparently was spread directly by contact to infect another stallion on the property (McCollum et al., 1999), after which it was efficiently transmitted horizontally by aerosol to other horses, perhaps because of an enhanced ability to replicate in susceptible cells within the respiratory tract. In contrast, many viral variants present in semen may be better adapted to persist in the reproductive tract of the stallion and replicate only poorly in the respiratory tract (Patton et al., 1999). Variants with the capacity to replicate efficiently in the respiratory tract would be expected to dominate during horizontal aerosol transmission, and so emerge as the dominant strain during an outbreak of EVA. Another plausible explanation for the genetic

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**Fig. 5.** Multiple alignment of the amino acid sequences encompassing amino acids 51–121 (which includes the V1 variable region (amino acids 61–121)) in the amino-terminal portion of the G protein of clones derived by RT–PCR from foal tissues and semen samples, as compared to the virus present in the semen of stallion A at the time of the outbreak (A2). Dots indicate identity with the A2 sequence. Potential N-linked glycosylation sites are shown in bold and underlined; *, stop codon; †, sequences that have deletions in this region or elsewhere.
homogeneity of EAV during the outbreak would be that the contact stallion was infected with a single variant by chance, and that this variant was amplified and subsequently circulated as the dominant strain during the outbreak.

It has been previously reported that selection of variants also occurs following experimental lactate dehydrogenase-elevating virus (LDV) infection of mice and during natural infection of monkeys with simian haemorrhagic fever virus (SHFV; Plagemann, 1996). LDV and SHFV are also arteriviruses (Plagemann, 1996). During epizootics of SHF in macaque monkeys, viral variants are selected that are virulent for both macaques as well as patas monkeys. In contrast, variants with lower virulence and immunogenicity are selected during persistent SHFV infection of patas monkeys. Our findings indicate that EVA outbreaks can be associated with selection of virulent variants from the EAV quasispecies generated during persistent infection of the carrier stallion.

Examination of multiple clones of viruses present in foal tissues and the semen of carrier stallions clearly indicates that the genetic heterogeneity of EAV during persistent infection in the stallion is considerably greater than that generated during this outbreak of EVA (Hedges et al., 1999). The presence of deletion mutant viruses in the semen of stallion P, and the rapid increase in micro-heterogeneity of viruses within the quasispecies, may have led to a highly unstable, unfit virus population (loss of population equilibrium) in the reproductive tract of this stallion. Generation of defective interfering particles could reflect either a mechanism responsible for virus persistence in the carrier stallion or more likely, a loss of virus fitness that contributed to the clearance of the virus from the reproductive tract of stallion P after one year of persistent infection (Domingo et al., 1998).

The variable glycosylation of the Gp, GP3, GP4 and G1 proteins observed in this study suggests that loss or acquisition of glycosylation sites in different EAV proteins might facilitate transmission and persistence of a virus that has a relatively limited capacity to generate genetic diversity. The loss of two N-glycosylation sites in the VP-3 envelope glycoprotein of LDV-C and LDV-v is associated with increased neurovirulence, whereas LDV-P has all three N-glycosylation sites in the VP-3 protein and replicates in macrophages during persistent infection without producing neurological signs (Anderson et al., 1995; Faaberg et al., 1995; Plagemann, 1996). Variable glycosylation of individual structural proteins, therefore, can influence the pathogenesis and virulence of arterivirus infections.

In summary, the nucleotide and phylogenetic analyses included in this study confirm the epidemiological data that virus present in the semen of imported carrier stallion A initiated the outbreak of EVA on this farm in Pennsylvania. The data indicate that a variant(s) of EAV that was present in the semen of stallion A was selected and efficiently transmitted horizontally by aerosol. Sequence data obtained from multiple clones derived from foal tissues and the semen of persistently infected stallions are consistent with the hypothesis that the carrier stallion can be a source of genetic and phenotypic diversity of EAV (Hedges et al., 1999), and that specific viral variants present in semen can be efficiently transmitted by aerosol to initiate outbreaks of EVA.

The authors would like to thank Dave Pettigrew for sequencing assistance. These studies were supported by the Grayson-Jockey Club Research Foundation, Inc., Lexington, Kentucky, USDA-NRI Competitive Grant 97-35204-4736 and the Center for Equine Health, University of California, Davis with funds provided by the Oak Tree Racing Association, the State of California Satellite Wagering Fund, and contributions by private donors.

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Received 22 January 1999; Accepted 9 April 1999