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Review

Experiences with infectious cDNA clones of equine arteritis virus: Lessons learned and insights gained

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ABSTRACT

The advent of recombinant DNA technology, development of infectious cDNA clones of RNA viruses, and reverse genetic technologies have revolutionized how viruses are studied. Genetic manipulation of full-length cDNA clones has become an especially important and widely used tool to study the biology, pathogenesis, and virulence determinants of both positive and negative stranded RNA viruses. The first full-length infectious cDNA clone of equine arteritis virus (EAV) was developed in 1996 and was also the first full-length infectious cDNA clone constructed from a member of the order *Nidovirales*. This clone was extensively used to characterize the molecular biology of EAV and other Nidoviruses. The objective of this review is to summarize the characterization of the virulence (or attenuation) phenotype of the recombinant viruses derived from several infectious cDNA clones of EAV in horses, as well as their application for characterization of the molecular basis of viral neutralization, persistence, and cellular tropism.

Contents

Historical perspective of putative "prototype strains" of equine arteritis virus used in various laboratories

Equine arteritis virus (EAV) was first isolated from the lung of an aborted fetus following an extensive outbreak of respiratory disease among horses on a Standardbred breeding farm in Bucyrus, Ohio in 1953 (Doll et al., 1957a, b). This original isolate was identified prior to the advent of routine cell culture; specifically, tissues collected from the aborted foal caused disease ("equine viral arteritis" [EVA]) in an experimentally inoculated horse, although the infection was not lethal (Doll et al., 1957a, b; Jones et al., 1957). This experimentally infected horse was euthanized and a homogenate of the animal's spleen was inoculated into another horse. The virus was then serially passaged 15 times

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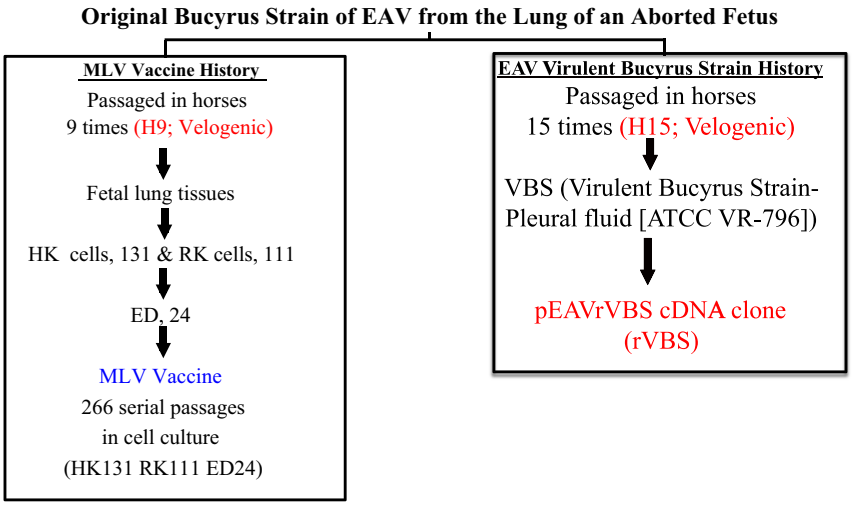

Fig. 1. Passage history of the parental virulent Bucyrus strain of EAV. (H = Passaged in horse; HK = Primary horse kidney cell, RK = Primary rabbit kidney cell passage, ED = Equine dermis cell [NBL-6; ATCC CCL-57]).

in horses by the late Dr. William H. McCollum and colleagues at the University of Kentucky (Fig. 1). The repeated serial passage of this original "Bucyrus virus" in its natural host markedly enhanced its virulence. The H9 (horse passage 9) and H15 (horse passage 15) strains of the passaged virus consistently caused severe clinical disease, and frequently death in horses (60% fatality rate; so-called "velogenic or virulent Bucyrus EAV") following experimental inoculation (MacLachlan et al., 1996; McCollum and Timoney, 1999). Following the creation of continuous cell lines, the H15 virus (pleural fluid archived by the late Drs. Roger Doll and William H. McCollum) was passaged in rabbit kidney (RK-13) cells and aliquots of this RK-13 passaged Bucyrus strain of EAV were deposited in the American Type Culture Collection (ATCC, Manassas, VA; catalog number VR-796). Significantly, many historic references to use of the putative "Bucyrus strain of EAV" do not, in fact, include the original virus associated with the 1953 EVA outbreak. Unfortunately, the original Bucyrus virus and the subsequent horse passage strains (up to 9th horse passage [H9]) have all been lost due to freezer failures. Only the archived pleural fluid from horse passage 15 (H15, also identified as EAV VB53 or virulent Bucyrus strain [VBS]) and the limited RK-13 passaged derivative of this virus (ATCC VR-796) now remain. Full-length genome sequence analysis of these two viruses (H15 and ATCC VR-796) in our laboratory has shown them to be 99.9% identical, and both cause fatal EVA in the majority of experimentally inoculated horses (MacLachlan et al., 1996; McCollum and Timoney, 1999). Thus, the pleural fluid from the original 15th horse passage and the ATCC VR-796 are now both designated as the VBS strain of EAV. Importantly, however, VBS is itself an artifact in that it causes severe, often fatal disease in horses, whereas field strains of EAV do not typically cause fatal disease in adult horses (Balasuriya et al., 2013 and references therein; Balasuriya and Maclachlan, 2004; McCollum and Timoney, 1999). Indeed, many field strains cause very mild disease or even subclinical infections (Balasuriya et al., 1999b, 2002, 2007; Balasuriya and MacLachlan, 2004; Go et al., 2012a; Patton et al., 1999; Pronost et al., 2010; Vairo et al., 2012; Zhang et al., 2010, 2012).

Drs. Doll and McCollum distributed the VBS strain of EAV to various laboratories in Europe and elsewhere during the late 1950s and thereafter. This original VBS virus was then extensively passaged *in vitro*, and sometimes plaque purified, in different laboratories. These extensively cell culture passaged, essentially laboratory-adapted EAV strains are often misleadingly designated as Bucyrus EAV without any reference to their passage history. Simply stated, these are highly cell culture passaged laboratory derivatives of the VBS strain of EAV, with distinctive genetic sequences and phenotypic properties. Finally, although the VBS strain of EAV is itself somewhat of a laboratory aberration due to its sequential experimental passage in horses, this virus is useful for studying virulence determinants of EAV and mechanisms of viral pathogenesis. Furthermore, the current modified live virus (MLV; live attenuated) EAV vaccine (ARVAC®, Zoetis, Kalamazoo, MI, USA) used for immunization of horses in the United States was originally produced by extensive cell culture passage of EAV VBS, as were laboratory strains such as EAV 030 from which the original infectious cDNA clone was derived (van Dinten et al., 1997) (Fig. 2).

Infectious cDNA clones of equine arteritis virus

It has long been known that positive-sense viral RNA is infectious and can generate progeny virus following its introduction into cells. Alexander et al. (1958a, b) first demonstrated the infectivity of poliovirus RNA in HeLa cells. Subsequently, Racaniello and Baltimore (1981a, b) developed the first infectious cDNA clone of poliovirus by cloning the full-length RNA genome into a bacterial plasmid vector. The advent of reverse transcription polymerase chain reaction (RT-PCR) technology in the mid-1980s, along with other recombinant DNA techniques, expedited the development of infectious cDNA clones of other RNA viruses (Boyer and Haenni, 1994; Mullis and Faloona, 1987). It was subsequently shown in numerous virus systems that *in vitro* transcripts of cDNA clones, and in some instances the cDNA itself, can initiate a complete productive infectious cycle in susceptible mammalian cells. As a result, genetic manipulation (reverse genetics) of full-length cDNA clones has become the most important tool to study the biology, pathogenesis, and virulence determinants of both positive and negative stranded RNA viruses. Reverse genetic strategies are especially useful for identification and functional characterization of specific viral genes because they demonstrate phenotypic effect(s)/consequences of introducing defined nucleotide change(s) to the gene of interest.

EAV is included within the order *Nidovirales*, and it is the prototype virus of the genus *Arterivirus*, family *Arteriviridae* (Fig. 3). Similar to other positive stranded RNA viruses, the genomes

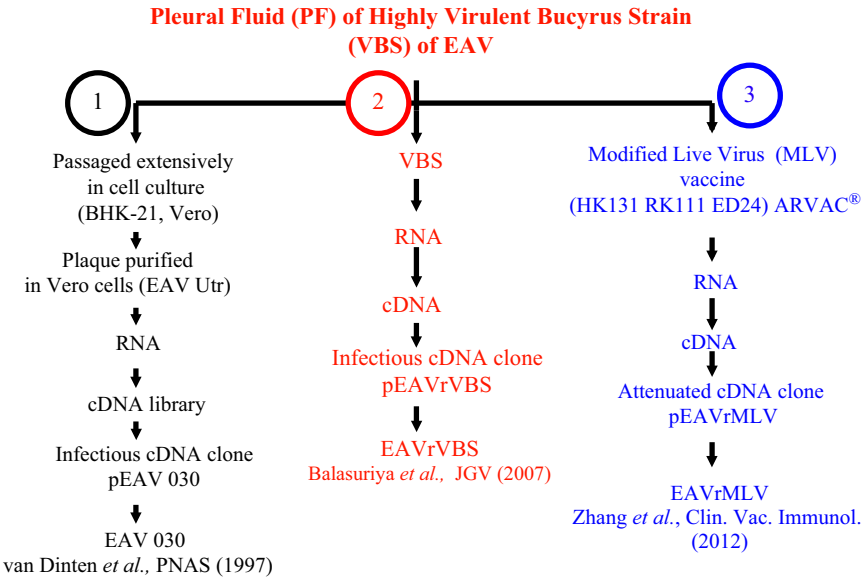

Fig. 2. Most commonly used infectious cDNA clones of EAV.

of Arteriviruses are infectious to cells (Meulenberg et al., 1993; van der Zeijst and Horzinek, 1975). The first full-length infectious cDNA clone of EAV was developed in 1996 by cloning twelve fragments from a cDNA library spanning the entire genome of a highly cell culture adapted laboratory strain of EAV downstream of the T7 RNA polymerase promoter in the pUC18 plasmid vector (pEAV 030 [GenBank accession number Y07862], Fig. 2) (van Dinten et al., 1997). This was also the first full-length infectious cDNA clone constructed from a member of the order *Nidovirales*. A second infectious cDNA clone of a very similar, highly cell culture-adapted laboratory strain of EAV was described soon thereafter (de Vries et al., 2000, 2001; Glaser et al., 1999). More recently, we have described the development of two infectious cDNA clones of EAV; the first from the highly-virulent, horse-adapted VBS strain of EAV (pEAVrVBS [DQ846751]) (Balasuriya et al., 2007) and the other from the MLV vaccine strain of EAV (ARVAC®, Zoetis, Kalamazoo, MI, USA, pEAVrMLV [FJ798195]) (Zhang et al., 2012) that was originally developed by extended cell culture passage of the VBS virus.

Eric Snijder and colleagues at Leiden University in the Netherlands have performed pioneering work using the pEAV 030 infectious cDNA clone to characterize the molecular biology of EAV and other Nidoviruses (Siddell et al., 2005; Snijder et al., 1995, 2005, 2013; Snijder and Spann, 2007; Snijder, 1998, and references therein; Snijder and Kikkert, 2013 and references therein). Specifically, they have used this infectious clone to better characterize EAV replication, including the processing of viral non-structural proteins (nsps) (Balasuriya et al., 2013; den Boon et al., 1995; Snijder, 1998, 2001; Snijder and Spann, 2007; van Aken et al., 2006a, b; van Dinten et al., 1999), mechanisms of subgenomic mRNA (sgmRNA) transcription (Tijms et al., 2001, 2007; Tijms and Snijder, 2003; van den Born et al., 2005; van Dinten et al., 2000; van Marle et al., 1999a, b), and the role of the viral replication complex (Snijder et al., 2006; Snijder, 2001). These studies have broad relevance and implications to the molecular characterization and replication strategies of all members of the order *Nidovirales*, including Coronaviruses, Toroviruses, and other Arteriviruses (lactate dehydrogenase elevating virus [LDV] of mice, porcine reproductive and respiratory syndrome virus [PRRSV], simian hemorrhagic fever virus [SHFV], and wobbly possum disease virus [WPDV]) (den Boon et al., 1991b; Dunowska et al., 2012; Gorbalenya et al., 2006; Kroese et al., 2008; Molenkamp et al., 2000; Pasternak et al., 2006; Siddell et al., 2005; Smits et al., 2006; Snijder et al., 2013). The small size of the EAV genome, as compared to those of Corona- and Toroviruses, has made it an ideal model system with which to study the replication strategy of Nidoviruses (Siddell et al., 2005; Smits et al., 2006). This work has been extensively reviewed previously (Snijder et al., 2001; Snijder and Spann, 2007; Snijder et al., 2013 and references therein, Snijder and Kikkert, 2013 and references therein), thus the objective of this article is to review our recent work using three infectious cDNA clones of EAV (pEAV 030, pEAVrVBS and pEAVrMLV) that are all ancestrally related to the parental VBS strain of EAV but which differ significantly in their virulence phenotypes (Fig. 2). Specifically, this review is focused on characterization of the virulence phenotype of the recombinant viruses derived from these infectious cDNA clones of EAV (EAV 030, EAV rVBS and EAV rMLV) in horses (Balasuriya et al., 1999b, 2007; Go et al., 2012b; Zhang et al., 2012), as well as their application to characterization of the molecular determinants of EAV neutralization, persistence, and protective host immunity.

Characterization of the genetic basis of attenuation of recombinant strains of EAV (EAV rVBS and EAV 030)

The virulence phenotype of recombinant viruses derived from the pEAVrVBS and pEAV 030 infectious cDNA clones of EAV (EAV rVBS and EAV 030, respectively) was characterized by experimental inoculation of horses (Balasuriya et al., 1999b, 2007; Go et al., 2012b) (Table 2). Horses inoculated with rVBS all developed severe clinical signs of EVA, including high fever, marked lymphopenia, petechial and ecchymotic hemorrhages in the oral mucous membranes, serous nasal discharge, supraorbital and limb edema of variable severity, and skin eruptions (hives). All horses developed high titer viremia with significant virus shedding in their nasal secretions. EAV was isolated for 28–56 days post-infection (DPI) from buffy coat, up to 12 DPI from plasma, and nasal shedding was detected until 10–12 DPI. Virus clearance coincided with the appearance of neutralizing antibodies in serum. However, in contrast, to the results of experimental infections with the original VBS strain, virus derived from the rVBS infectious clone did not cause fatal disease in

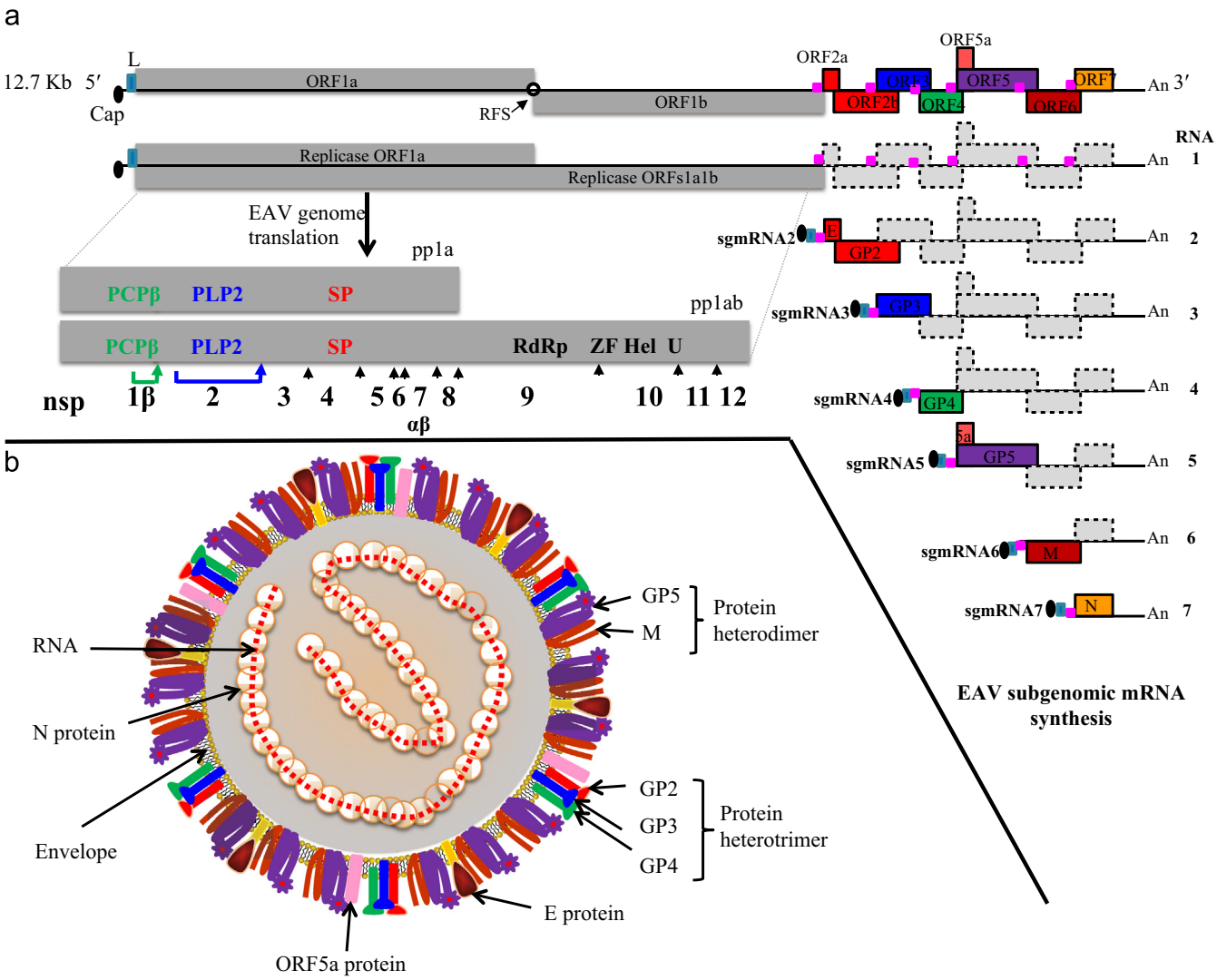

Fig. 3. EAV genome organization (a) and virion architecture (b). (a) The genomic open reading frames (ORFs) are indicated and the names of the corresponding proteins are depicted. The pink boxes represent the body transcription regulatory sequences (TRSs). The papain-like cysteine protease (PCPβ), papain-like protease domain 2 (PLP2 [previously known as cysteine protease; CP] that is predicted to contain the ovarian tumor domain-containing [OTU] superfamily of deubiquitinating enzymes [DUBs] on the basis of comparative sequence analysis) and serine protease (SP) are located in the nsp1, nsp2 and nsp4 of viral replicase, respectively. The nested set of mRNAs that is found in infected cells is depicted below the genome, with RNA1 being identical to the viral genome and sgmRNAs 2 to 7 being used to express the structural protein genes located in the 3′-proximal quarter of the genome. The light blue box at the 5′ end of each sgmRNA represents the common leader sequence, which is derived from the 5′ end of the genome. With the exception of the bicistronic sgmRNAs 2 and 5, the sgmRNAs are functionally monocistronic. Translation of proteins from sgmRNAs 2 (E and GP2 proteins) and 5 (ORF5a protein and GP5) by leaky scanning of the 5′-proximal end of these sgmRNAs (Firth et al., 2011; Snijder et al., 1999). The ORFs 1a and 1b located at the 5′ end of the genome are translated into two polyproteins (pp1a and pp1ab) that are further processed into 12–13 nonstructural proteins by three viral proteases (nsps 1, 2, and 4). (b) EAV particle consists of a nucleocapsid (N) and seven envelope proteins which include two major envelope proteins (GP5 and M form a dimer), three minor envelope glycoproteins (GP2, GP3, and GP4 form a trimer), and two other minor envelope proteins (E and ORF5a protein). Adapted from Balasuriya et al. (2013) with permission.

horses. Comparative nucleotide sequence analysis of the original VBS virus (GenBank accession number DQ846750) and rVBS (DQ846751) showed 99.9% identity, with only 6 coding differences between the two viruses. Five of these amino acid differences are located in non-structural proteins (nsp2 [2], nsp9 [1] and nsp10 [2]) and the sixth amino acid change is present in the major envelope glycoprotein (GP5 [1]) (Tables 3 and 4). These data strongly suggest that major virulence determinants leading to fulminant EVA in horses (characterized by disseminated intravascular coagulopathy and death) are localized to one or more key amino acid residues located in nsp2 (382 Gly→Asp and 559 Asn→Ser), nsp9 (1970 Asp→Gly), nsp10 (2400 Val→Ala and 2657 Ser→Cys), and GP5 (104 Asp→Asn). These amino acid changes that were inadvertently introduced during the cloning process clearly had a significant impact (attenuation) on the virulence phenotype of the parental VBS strain of EAV.

We have also previously evaluated the virulence phenotype of the recombinant virus derived from the pEAV 030 infectious cDNA clone (EAV 030) by experimental inoculation of two stallions (Balasuriya et al., 1999b). Neither stallion developed significant clinical manifestations of EVA, rather both had mild transient fever and mild to moderate lymphopenia (Table 2). Virus was isolated transiently from nasal swabs and mononuclear cells collected from both stallions. Virus was also isolated from the semen of one stallion at 7 DPI, but neither stallion became persistently infected with EAV. These data confirm that the recombinant EAV 030 virus is highly attenuated as compared to VBS and its recombinant counterpart (EAV rVBS). Importantly, although designated as a prototype strain of EAV, EAV 030 is a laboratory variant that was derived by extensive cell culture passage of VBS as well as its cloning by end point dilution and plaque purification in African green monkey cells (Vero cells; see above and Fig. 2). Viral RNA from

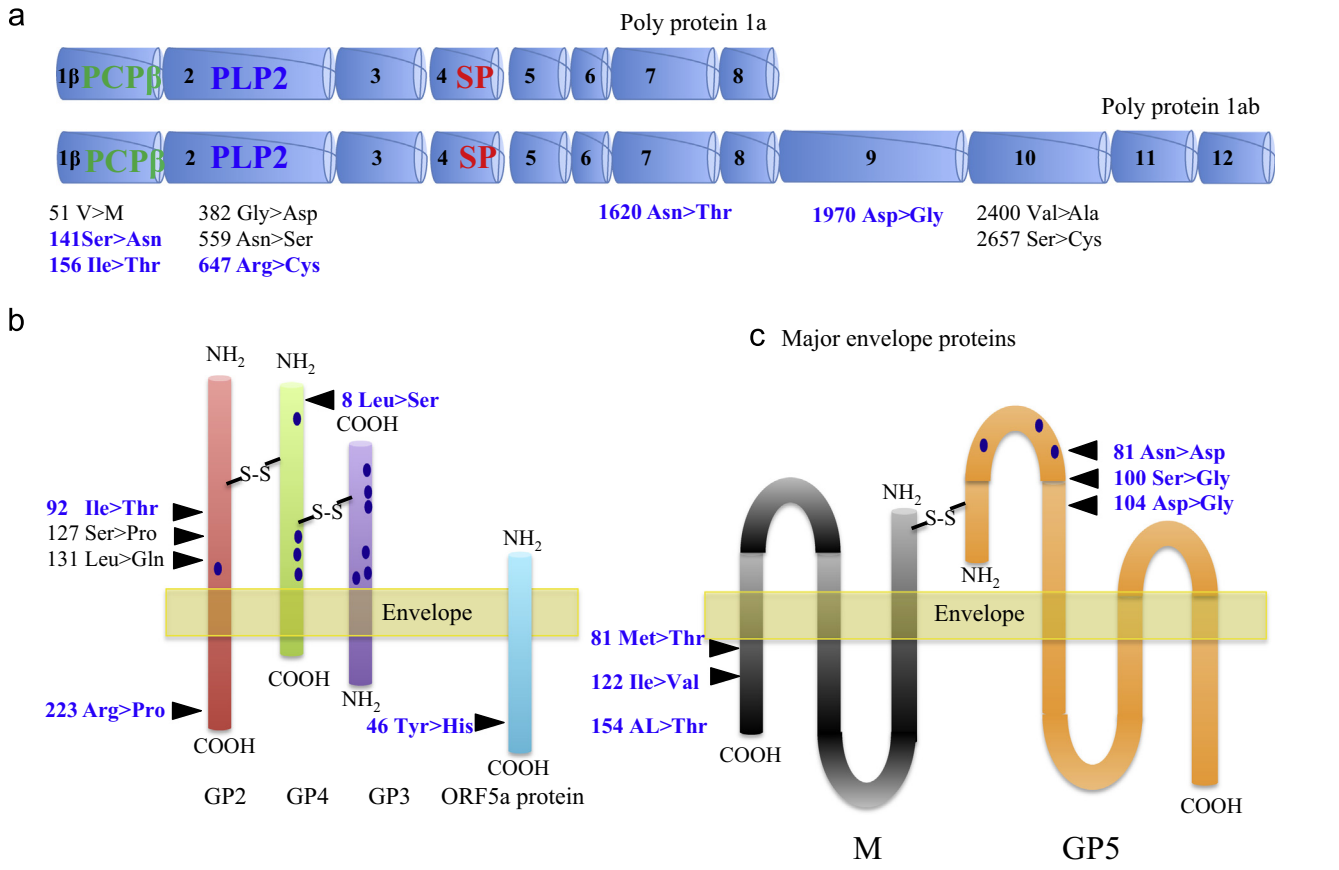

Fig. 4. Comparative amino acid sequence analysis between the EAV VBS and attenuated EAV strains derived from it identified several amino acid substitutions in both non-structural (A) and structural proteins (B and C). The putative glycosylation sites in GP2 (Asn-155), GP3 (Asn-28, Asn-29, Asn-49, Asn-96, Asn-106, and Asn-118), GP4 (Asn-33, Asn-55, Asn-65, and Asn-90), and GP5 (Asn-56, Asn-71, and Asn-81) are depicted in blue dots. The most significant amino acid changes are highlighted (blue and bold) in the figure.

Table 1
Sequential passage history of EAV virulent Bucyrus strain en route to development of the MLV vaccine strain of EAV.

Horse/cell culture passage of the virus	*In vivo* studies (virulence/clinical signs)	Reference
EAV Virulent Bucyrus Strain (EAV VBS; ATCC-VR-796)[a]	Velogenic – severe fatal disease (60% fatality rate), abortion	MacLachlan et al. (1996), McCollum and Timoney (1999)
EAV HK25[b]	Mesogenic – less severe disease (40.4°C body temperature, severe leukopenia)	McCollum et al. (1961b), McCollum (1969)
EAV HK116[b]	Lentogenic – no clinical signs and horses developed immunity to EAV	McCollum et al. (1962), McCollum (1969, 1970a, b)
EAV HK131 RK111 ED24 [ARVAC® MLV vaccine][b]	Lentogenic – no clinical signs and horizontal transmission in inoculated horses. Intramuscular administration – horses developed protective immunity and did not transmit the virus to contact animals. Intranasal inoculation – did not immunize effectively.	Harry and McCollum (1981), McKinnon et al. (1986), Timoney et al. (1988, 2007)

H=Passaged in horse; HK=primary horse kidney cell, RK=primary rabbit kidney cell passage, and ED=equine dermis cell (NBL-6; ATCC CCL-57).

[a] H9, H10 or H15 (Horse passage 9, 10 or 15).

[b] Each virus is identified by the cell type followed by the number of passages in that particular cell type (HK25 and HK116 – EAV VBS was serially passaged 25 and 116 times respectively in primary horse kidney cells; HK131RK111ED24 – EAV VBS was serially passaged in primary horse kidney cells for 131 times, primary rabbit kidney cells for 111 times, and equine dermis cell line for 24 times).

this highly cell culture adapted laboratory strain of EAV was used to generate the genomic cDNA library that was used to construct the original infectious cDNA clone of EAV 030 (Balasuriya et al., 1999b; van Dinten et al., 1997). Most probably, plaque purification and cell culture propagation of the original virus subjected it to a genetic bottleneck that led to the selection of an attenuated variant. The comparative nucleotide and amino acid sequence analysis between EAV 030 and the original VBS strain identified 9 amino acid changes in nsps (nsp1 [4], nsp2 [3], nsp9 [1], and nsp10 [1]) and 12 in structural proteins (GP2 [2], GP4 [4], ORF5a protein [1], GP5 [3], and M [2]) (Tables 3 and 4 and Fig. 4). Of these, two amino acid changes in nsp9 and nsp10 were common to both EAV rVBS and EAV 030 and appear to have a significant effect on the attenuation of these two recombinant viruses. Furthermore, substitution of 81Asn→Asp in the GP5 protein of EAV 030 led to the loss of a glycosylation site that might also contribute to attenuation of the virus.

Characterization of the genetic basis of attenuation of a modified live virus (MLV) vaccine strain of EAV

With the advent of cell culture systems, the VBS strain of EAV (H9) was serially passaged in primary equine kidney (HK), primary rabbit kidney (RK), and equine dermal (ED; NBL-6; ATCC CCL57) cells to attenuate the virus to create a MLV vaccine (ARVAC®; Fig. 1). Viruses at different levels of cell culture passage have been inoculated previously into horses to characterize their virulence phenotype (Table 1) (Doll et al., 1968; Harry and McCollum, 1981; McCollum, 1969, 1970a, b; McCollum et al., 1961a, b, 1962; McCollum and Timoney, 1999; McKinnon et al., 1986; Summers-Lawyer et al., 2011; Timoney, 1988; Timoney et al., 2007; Zhang et al., 2012). The viruses span the entire spectrum of attenuation of the highly virulent horse-adapted VBS strain of EAV to the completely attenuated MLV (ARVAC®) vaccine derived from it (Doll et al., 1968; Harry and McCollum, 1981; McCollum, 1969, 1970a, b; McCollum et al., 1961a, b, 1962; McCollum and Timoney, 1999; McKinnon et al., 1986; Timoney et al., 1988). Comparative whole genome sequence analysis of viruses at different levels of attenuation (including VBS, a moderately virulent [mesogenic; HK-25] and two avirulent [lentogenic; HK116 and HK131/RK-111ED24; ARVAC® vaccine] strains) identified a limited number of potential attenuating mutations (Tables 3 and 4). Interestingly, none of these differences are located in either the 5′ or 3′ UTR, rather they were located only in genes encoding various structural and non-structural proteins.

Following 25 passages in HK cells, the HK25 virus had a moderately virulent (mesogenic) phenotype in horses and comparative amino acid sequence analysis identified 12 amino acid changes as compared to the VBS strain. Seven of these amino acid changes were in nsps (nsp1 [4], nsp3 [1], nsp7 [1], and nsp9 [1]) and five were in structural proteins (GP4 [1], ORF5a protein [1], GP5 [1], and M [2]; Tables 3 and 4). Subsequent passages in HK cells further attenuated the virus and by the 116th passage (HK116), it was completely attenuated in horses. Comparative amino acid sequence analysis showed nine additional amino acid changes as compared to the mesogenic HK25 virus (total of 17 amino acid changes as compared to the VBS strain; Tables 3 and 4). These include four amino acid changes in nsps (nsp2 [1], nsp3 [1], and nsp7 [2]; Table 3), and five additional amino acid substitutions in structural proteins (GP2 [2], GP5 [2], and M [1]; Table 4). In summary, comparison of the virulence phenotype in horses and sequences of the VBS, HK25, and HK116 viruses identified just 16 amino acid substitutions that individually or collectively appear to be responsible for attenuation of the HK116 virus. These include six amino acid substitutions in the replicase proteins (nsp1: Val51→Met, Ser141→Asn, Ile156→Thr; nsp2: Arg647→Cys; nsp7: Asn1620→Thr; and nsp 9 Asp1970→Gly) and 10 amino acid substitutions in the structural proteins (GP2: Ile92→Thr, Arg223→Pro; GP4: Leu8→Ser; ORF5a protein: Tyr46→His; GP5: Asn81→Asp, Ser100→Gly, Asp104→Gly; and M: Met81→Thr, Ile122→Val, Ala154→Thr) of EAV (Tables 3 and 4).

Interestingly, some of the amino acid substitutions acquired during sequential cell culture passage of EAV 030 (BHK-21) and HK116 (primary horse kidney) virus strains were identical, further suggesting that these common amino acid changes play an important role in attenuation of the virulent VBS virus during sequential cell culture passage. Both attenuated viruses had amino acid substitutions in nsp1 (Val51→Met, Ser141→Asn, and Ile156→Thr), nsp9 (Asp1970→Gly), GP4 (Leu8→Ser), GP5 (Asn81→Asp), and M (Ile122→Val, Ala154→Thr). Attenuation of the VBS strain of EAV to the mesogenic HK25 virus involves a single amino acid change (Asn81→Asp) that results in a loss of the glycosylation site at position 81 in the GP5 protein. This substitution was conserved among all of the cell culture adapted viruses. The variable retention of the glycosylation site at position 81 in the GP5 protein among field strains of EAV further suggests this residue might play a central role in EAV virulence and pathogenesis (Balasuriya et al., 1997, 1998, 1999a, 2004b; Zhang et al., 2008b, 2010). Similarly, the loss of glycosylation sites of the equivalent LDV protein (VP-3P) alters both virulence and the cellular tropism of the virus (Chen et al., 2000; Plagemann, 2001a; Plagemann et al., 2001b, c). Moreover, most of the amino acid substitutions in the GP5 protein were located in three (B, C, and D) of the four major neutralization sites (Balasuriya et al., 2013 and references therein). Unequivocal determinations of the significance of individual nucleotide and amino acid substitutions were further determined in prospective studies using reverse genetic manipulation of infectious cDNA clones (see below).

To further confirm the significance of 14 amino acid substitutions identified by comparative sequence analysis in three nsps

Table 2
Virulence phenotype of the prototype VBS of EAV and various recombinant viruses derived from the infectious cDNA clones.

Virus strain	Virulence phenotype	Clinical signs/necropsy findings[a]	Reference
EAV VBS (H15)/ATCC VR-796	Velogenic[b] – fatal disease in adult horses	Severe fatal disease (lethargy and anorexia; 5–8 DPI[d] high fever 39.1–40.3 °C; ventral and dependent edema; periocular edema; serous nasal discharge; petechial hemorrhages in the oral mucosa; death 9 DPI). Necropsy (several liters of straw-colored transudate in the pleural cavity and extensive petechial and ecchymotic hemorrhages on the serosa of the bowel). High-titer viremia (1.1×10^3–1.1×10^4 $TCID_{50}$/50 μl, 3–8 DPI). Neutralizing antibodies appear 6–8 DPI.	MacLachlan et al. (1996), McCollum and Timoney (1999)
EAV rVBS	Mesogenic[b] – less severe disease	Moderate to severe disease (depression; 5–6 DPI high fever 38.9–40.6 °C [3–9 DPI]; supraorbital and limb edema; severe lymphopenia [4–8 DPI]; serous nasal discharge [6–9 DPI]; petechial and ecchymotic hemorrhages in the oral mucosa [7–8 DPI]; skin eruptions (hives) on the neck, shoulder, along the back: High-titer viremia (6×10^3–1×10^5 PFU/ml; [4–10 DPI]); virus isolated from buffy coat for 28–56 DPI; nasal shedding of virus (4 and 6 DPI, virus titer 4×10^4 PFU/ml). Neutralizing antibodies appear 6–8 DPI and increased to >512 by 10–14 DPI.	Balasuriya et al. (2007), Go et al. (2012b)
EAV 030	Lentogenic[b] – mild transient clinical signs	Mild, transient fever 39.1 °C; mild to moderate lymphopenia; mild viremia ($\leq1\times10^1$ PFU/ml [2–14 DPI]; nasal shedding ($\leq1\times10^1$ PFU/ml [2–14 DPI]. Neutralizing antibodies appear 10 DPI and increased to 64–256 by 14–42 DPI.	Balasuriya et al. (1999a, 1999b)
EAV rMLV	Lentogenic[c] – no clinical signs	No fever; mild transient viremia (<1–2 PFU/ml in a few horses [2 DPI]); transient nasal shedding (2 PFU/ml in a few horses [2 DPI or 4 DPI or 4–6 DPI]).	Zhang et al. (2012)

[a] Fatal disease.
[b] Following intranasal inoculation.
[c] Following intramuscular inoculation.
[d] DPI – days post infection.

Table 3
Comparative amino acid sequence analysis of non-structural proteins between VBS of EAV and two recombinant viruses (EAV rVBS and EAV 030) and three cell culture passaged EAV strains (HK25, HK116 and ARVAC MLV vaccine).

Open Reading Frames (ORFs)	Protein (aa length)	Amino acid substitutions[a]				Amino acid substitutions[a]		
		Amino acid position	EAV VBS (DQ846750)	EAV rVBS (DQ846751)	EAV 030 (Y07862)	EAV HK25 (EU586273)	EAV HK116 (EU586274)	ARVAC (EU586275)
ORF1ab (225–9751)	**Nonstructural proteins (nsp) 1ab polyprotein (3175 aa length)**							
	nsp1: Met1-Gly260 (260)	9	Phe	Phe	Phe	Leu	Phe/Leu[b]	Phe
		51	Val	Val	Met	Met	Met	Val
		141	**Ser**	**Ser**	**Asn**	**Asn**	**Asn**	**Asn**
		145	Ala	Ala	Val	Ala	Ala	Val
		156	**Ile**	**Ile**	**Thr**	**Thr**	**Thr**	**Thr**
	nsp2: Gly261-Gly831 (571)	353	Thr	Thr	Ile	Thr	Thr	Thr
		382	Gly	Asp	Gly	Gly	Gly	Gly
		388	Thr	Thr	Thr	Thr	Thr	Ile
		402	Arg	Arg	Arg	Arg	Arg	His
		404	Ser	Ser	Ser	Ser	Ser	Cys
		429	Lys	Lys	Thr	Lys	Lys	Lys
		500	Leu	Leu	Leu	Leu	Leu	His
		525	Val	Val	Val	Val	Val	Met
		559	Asn	Ser	Asn	Asn	Asn	Asn
		579	Gln	Gln	Gln	Gln	Gln	His
		647	**Arg**	**Arg**	**Arg**	**Arg**	**Cys**	**Cys**
		731	Ile	Ile	Thr	Ile	Ile	Ile
	nsp3: Gly832-Glu1064 (233)	873	Thr	Thr	Thr	Thr	Thr	Ile
		957	Ala	Ala	Ala	Thr	Ala	Ala
	nsp4: Gly1065-Glu1268 (204)	–[†]	–	–	–	–	–	–
	nsp5: Ser1269-Glu1430 (162)	1372	Met	Met	Met	Met	Met	Leu
	nsp6: Gly1431-Glu1452 (22)	–	–	–	–	–	–	–
	nsp7: Ser1453-Glu1677 (225)	1486	Ala	Ala	Ala	Val	Ala	Ala
		1620	**Asn**	**Asn**	**Asn**	**Asn**	**Thr**	**Thr**
	nsp8: Gly1678-Asn1727 (50)	–	–	–	–	–	–	–
	nsp9: Gly1678-Glu2370 (693)	**1970**	**Asp**	**Gly**	**Gly**	**Gly**	**Gly**	**Gly**
		2028	Lys	Lys	Lys	Lys	Lys	Arg
	nsp10: Ser2371-Gln2837 (467)	2400	Val	Ala	Ala	Val	Val	Val
		2449	Ile	Ile	Ile	Ile	Ile	Val
		2657	Ser	Cys	Ser	Ser	Ser	Ser
	nsp11: Ser2838-Glu3056 (219)	2948	Ala	Ala	Ala	Ala	Ala	Val
		2992	Asp	Asp	Asp	Asp	Asp	Gly
	nsp12: Gly3057-Val3175 (119)	–	–	–	–	–	–	–

†=No amino acid changes occurred.
Critical amino acid changes are identified in bold. All these changes are tested/confirmed by reverse genetics. Other major amino acid changes are identified in color.
[a] Amino acid substitutions as compared to the parental EAV VBS.
[b] Phe/Leu=Phe or Leu.

(nsp1, nsp2, and nsp7) and four structural proteins (GP2, GP4, GP5, and M) to attenuation, we introduced these substitutions into the virulent infectious cDNA clone (pEAVrVBS) that was derived from the VBS strain (DQ846751) (Zhang et al., 2008a). Site-directed mutagenesis was used to individually introduce the four amino acid substitutions in the nsps 1, 2, and 7 of the pEAVrVBS infectious clone (the new recombinant virus was identified as rVBS/Vacc NS4m), and the ten amino acid substitutions contained in the structural proteins (GP2, GP4, GP5, ORF5a protein, and M) of the HK116 virus were introduced by swapping ORFs2-7 of the HK116 virus in their entirety into the pEAVrVBS clone (chimeric rVBS/HK116 S virus; Tables 3 and 4). Horses inoculated with the chimeric rVBS/HK116 S virus developed only mild serous nasal discharge whereas some of the horses inoculated with rVBS/Vacc NS4m developed typical clinical signs of EVA, confirming that this virus is less attenuated than rVBS/HK116 S (Table 7). Since both recombinant viruses share the backbone of the virulent pEAVrVBS infectious clone, the virulence phenotypes of the rVBS/HK116 S and rVBS/Vacc NS4m viruses were also compared to that of the virulent parental rVBS virus. The average body temperature of the rVBS-inoculated horses was significantly higher than that of horses inoculated with either the rVBS/HK116 S or rVBS/Vacc NS4m viruses. Whereas nasal virus shedding and viremia were similar between rVBS- and rVBS/VaccNS4m-inoculated horses, both nasal shedding of virus and viremia in rVBS/HK116 S-inoculated horses were significantly lower than those in horses inoculated with either the rVBS- or rVBS/Vacc NS4m viruses. In summary, the rVBS/HK116 S virus had substantially reduced virulence for horses as compared to the parental rVBS strain, and the rVBS/Vacc NS4m virus caused only subclinical infection. Taken together these data suggest that amino acid changes in either the replicase (nsp1, nsp2 and nsp7) or structural proteins (GP2, GP4, GP5 and M) led to attenuation, but the recombinant virus with multiple substitutions in the structural proteins was more attenuated than the recombinant virus with substitutions only in the replicase proteins. The significance of the numerous non-coding

Table 4
Comparative amino acid sequence analysis of structural protiens between VBS of EAV and two recombinant viruses (EAV rVBS and EAV 030) and three cell culture passaged EAV strains (HK25, HK116 and ARVAC® MLV vaccine).

Open Reading Frames (ORFs)	Structural protein (aa length)	Amino acid substitutions[a]				Amino acid substitutions[a]		
		Amino acid position	EAV VBS (DQ846750)	EAV rVBS (DQ846751)	EAV 030 (Y07862)	EAV HK25 (EU586273)	EAV HK116 (EU586274)	ARVAC® (EU586275)
ORF2a (9751–9954)	**E** (67)	–**	–	–	–	–	–	–
ORF2b (9824–10507)	**GP2** (227)	62	Tyr	Tyr	Tyr	Tyr	Tyr	His
		92	**Ile**	**Ile**	**Ile**	**Ile**	**Thr**	**Thr**
		127	Ser	Ser	Pro	Ser	Ser	Ser
		131	Leu	Leu	Gln	Leu	Leu	Leu
		158	Gly	Gly	Gly	Gly	Gly	Glu
		223	**Arg**	**Arg**	**Arg**	**Arg**	**Pro**	**Pro**
ORF3 (10306–10797)[b]	**GP3** (163)[b]	80	Leu	Leu	Leu	Leu	Leu	Val
		123	Leu	Leu	Leu	Leu	Leu	Ser
		160	Cys	Cys	Cys	Cys	Cys	Tyr
		164	Stop	Stop	Stop	Stop	Stop	Gln
		165						Phe
		166						Tyr
		167						Leu
		168						His
		169						Stop
ORF4 (10700–11158)	**GP4** (152)	4	Tyr	Tyr	Tyr	Tyr	Tyr	Ser
		8	**Leu**	**Leu**	**Ser**	**Ser**	**Ser**	**Ser**
		29	Ala	Ala	Ala	Ala	Ala	Thr
		37	Ile	Ile	Ile	Ile	Ile	Thr
		69	Leu	Leu	Pro	Leu	Leu	Leu
		115	Asn	Asn	Asp	Asn	Asn	Asn
		149	Gln	Gln	Arg	Gln	Gln	Gln
ORF5a (11112–11291)	**ORF5a protein** (59)	12	Asn	Asn	Asp	Asn	Asn	Asn
		13	Val	Val	Val	Val	Val	Ala
		19	Ala	Ala	Ala	Ala	Ala	Val
		46	**Tyr**	**Tyr**	**Tyr**	**His**	**His**	**His**
ORF5 (11146–11913)	**GP5** (255)	69	Leu	Leu	Leu	Leu	Leu	Pro
		72	Gln	Gln	Gln	Gln	Gln	Lys
		81	**Asn**	**Asn**	**Asp**	**Asp**	**Asp**	**Asp**
		100	**Ser**	**Ser**	**Ser**	**Ser**	**Gly**	**Gly**
		101	Val	Val	Ala	Val	Val	Val
		104	**Asp**	**Asn**	**Asp**	**Asp**	**Gly**	**Gly**
		106	Met	Met	Met	Met	Met	Val
		170	Ala	Ala	Ala	Ala	Ala	Ser
		214	Gly	Gly	Gly	Gly	Gly	Glu
		220	Ala	Ala	Val	Ala	Ala	Ala
ORF6 (11901–12389)	**M** (162)	38	Leu	Leu	Leu	Leu	Leu	Ser
		49	Phe	Phe	Phe	Phe	Phe	Leu
		71	Val	Val	Val	Val	Val	Ala
		81	**Met**	**Met**	**Met**	**Met**	**Thr**	**Thr**
		122	**Ile**	**Ile**	**Val**	**Val**	**Val**	**Val**
		150	Phe	Phe	Phe	Phe	Phe	Cys
		154	**Ala**	**Ala**	**Thr**	**Thr**	**Thr**	**Met**
ORF7 (12313–12645)	**N** (110)	13	Phe	Phe	Phe	Phe	Phe	Val
		17	Arg	Arg	Arg	Arg	Arg	Trp
		104	Val	Val	Val	Val	Val	Ile
Total number of amino acid substitutions compared to the EAV VBS (non-structural and sturcural proteins [Tables 3 and 4])				**6**	**21**	**12**	**17**	**55**

**=No amino acid changes occurred.
Critical amino acid changes are identified in bold. All these changes are tested/confirmed by reverse genetics. Other major amino acid changes are identified in color.
[a] Amino acid substitutions as compared to the parental EAV VBS.
[b] In ARVAC® MLV vaccine strain, the ORF3 spans from nucleotides 10,306–10,812 and the GP3 is 168 amino acids in length.

mutations that occurred during cell culture passage of the VBS virus was not directly evaluated by reverse genetics.

To produce the current commercial MLV vaccine of EAV, the HK116 virus was further passaged 15 times in HK cells, followed respectively by 111 and 24 passages in RK and ED cells. These additional cell culture passages fixed all of the amino acid substitutions present in the HK116 virus, together with 43 additional amino acid substitutions as compared to HK116, including 15 in the nsps and 28 (including a 5 aa insertion) in the structural proteins (Zhang et al., 2008a). However, the numerous additional amino acid substitutions that accumulated during further cell culture passage of HK116 to HK131RK111ED24 (ARVAC® seed virus) did not alter the attenuation phenotype of the virus significantly as the virus was already fully attenuated by HK passage 116 (McCollum, 1969, 1970a, b; McCollum et al., 1962). However, this extensive additional cell culture passage did alter key neutralization epitopes

Table 5
Amino acid differences between the VBS and HeLa-H-EAVP80 viruses.

Open Reading Frames (ORFs)	Protein (aa length)[b]	Amino acid substitutions[a]		
		Position	EAV VBS	HeLa-H-EAVP80
ORF1ab (225–9751)	**Nonstructural proteins (nsp)**			
	1ab polyprotein (3175)			
	nsp1: Met1-Gly260 (260)	145	Ala	Val
	nsp2: Gly261-Gly831 (571)	577	Asp	Gly
	nsp7: Ser1453-Glu1677 (225)	1599	Lys	Arg
	nsp8/9: Gly1678-Asn1727 (50)/Glu2370(693)	1933	Pro	Ser
	Structural proteins			
ORF2a (9751–9954)	**E** (67)	53	Ser	Cys
		55	Val	Ala
ORF2b (9824–10507)	**GP2** (227)	15	Leu	Ser
		31	Trp	Arg
		87	Val	Leu
		112	Ala	Thr
ORF3 (10306–10797)	**GP3** (163)	115	Ser	Gly
		135	Leu	Pro
ORF4 (10700–11158)	**GP4** (152)	4	Tyr	His
		109	Ile	Phe
ORF5 (11146–11913)	**GP5** (255)	9	Phe	Ser
		98	Pro	Leu

[a] Amino acid substitutios are numbered according to the published sequence of EAV 030 virus (GenBank accession no. NC_002532).
[b] Amino acids of non-structural proteins are numbered according to their locations in the replicase polyprotein pp1ab. Amino acids of structural proteins are numbered according to their locations in individual structural protein. All these changes are tested/confirmed by reverse genetics (see Fig. 5).

located in the GP5 protein and significantly changed the neutralization phenotype of the MLV vaccine virus (see below; (Balasuriya et al., 1997, 2013; Balasuriya and MacLachlan, 2004; Zhang et al., 2010). Previous studies have shown that antibodies generated in horses (polyclonal equine antisera) against the MLV vaccine strain do not neutralize some field strains of EAV as effectively as equine antisera generated against the VBS strain (Balasuriya et al.,1998; Balasuriya et al., 2004b; Balasuriya et al., 2004a; Zhang et al., 2010) and, thus, excessive cell culture passage has perhaps overly attenuated this MLV vaccine virus and potentially compromised its protective efficacy.

Taken together, comparative amino acid sequence data analysis of EAV strains of different virulence to horses and the MLV vaccine strain confirm that the virulence determinants of EAV may be located in genes encoding both non-structural (nsp1 [contains a papain-like-cysteine protease domain that plays a major role in virus replication, sgmRNA synthesis, and virus production, and coordinates the viral replicative cycle], nsp2 [contains a papain-like protease domain 2; PLP2 and possesses deubiquitinating enzyme activity that antagonizes the host innate immune response], nsp9 [contains a RdRP domain and directs viral RNA synthesis in conjunction with other viral and cellular proteins], possibly nsp10 [contains a predicted zinc-binding domain; ZBD] in its N-terminus and a nucleoside triphosphate-binding/helicase (Hel) motif in its C-terminal domain (den Boon et al., 1991a) and both minor (GP2, GP4, and ORF5a protein) and major (GP5 and M) envelope proteins (Tables 3 and 4 and Fig. 4). Interestingly, viruses with very different cell culture passage histories share multiple common amino acid substitutions, confirming their likely importance in determining the virulence phenotype of EAV. Thus, it appears that attenuation of EAV is a complex process that potentially involves a variety of structural and nonstructural viral proteins, and perhaps the interaction of different proteins. Furthermore, these studies also identified several lethal amino acid substitutions that cause *in vitro* transcribed RNA to be noninfectious (no genomic replication or infectious progeny virus production) following transfection into mammalian cells. These include Ser1022→Leu (nsp3) (Zhang et al., 2012), Ser1453→Arg (nsp7) (Balasuriya et al., 2000) and Ser2429→Pro (nsp10) (van Dinten et al., 1997). The Ser1022→Leu substitution is located in the cytoplasmic tail of nsp3 and it is not clear how this mutation adversely affected virus replication. The arginine substitution at position 1453 affected the cleavage of nsp7 from nsp6 (Glu1452 [nsp6]/Ser1453 [nsp7]), which made the *in vitro* transcribed IVT RNA noninfectious following transfection. The Ser2429→Pro substitution located in the ZBD of nsp10 may affect multiple functions, specifically viral RNA replication, sgmRNA transcription, and biogenesis that may lead noninfectious RNA resulting in non-viable viruses.

Characterization of the genetic basis of persistent EAV infection

Some 10–70% of stallions infected with EAV subsequently become persistently infected carriers that continuously shed the virus in their semen (Timoney and McCollum, 1993). Persistently infected stallions are the principal reservoir of EAV and are responsible for perpetuation and dissemination of EAV in equine populations (Balasuriya and MacLachlan, 2004 Timoney and McCollum, 1993). Carrier stallions are also a significant natural source of genetic and phenotypic diversity of EAV (Balasuriya et al., 1999a, 2004b; Hedges et al., 1999). However, the viral factors involved in the establishment and maintenance of EAV persistence in the stallion are not well characterized. To identify the viral proteins involved in establishment of EAV persistence, we developed an *in vitro* model in HeLa-H (passage 170–222) cells (Zhang et al., 2008c). The VBS virus established persistent infection in HeLa-H cells and the virus recovered from the 80th passage of the persistently infected HeLa-H cells (HeLa-H-EAVP80) readily established persistent infection in the HeLa-L cells (passage 95–107), whereas the original VBS virus did not establish persistent infection in HeLa-L cells. Comparative nucleotide sequence analysis identified 33 nucleotide differences between the VBS and HeLa-H-EAVP80 viruses and no nucleotide differences were identified in the 5′ or 3′ UTR or ORFs 6–7. Approximately 50% of the nucleotide changes were silent and there were only 16 amino acid differences between the two viruses, including four in the replicase (nsp1, nsp2, nsp7, and nsp9) and 12 in the structural proteins (E, GP2,

GP3, GP4, and GP5; Table 5). The role of these amino acid changes in establishment of persistent EAV infection *in vitro* was further evaluated in a prospective study using reverse genetic technology. The recombinant rVBS/P80 NS4m virus, which has the identical sequence to the rVBS virus with the exception of four amino acid substitutions in the replicase polyprotein (nsp1 Ala145→Val, nsp2 Asp577→Gly, nsp7 Lys1559→Arg, and nsp9 Pro1933→Ser), was unable to establish persistent infection in the HeLa-L cell line (Fig. 5) whereas the recombinant virus rVBS/P80S, which carries the replicase gene of the parental rVBS and the structural protein genes of the HeLa-H-EAVP80 virus, did establish persistent infection in the HeLa-L cell line. This clearly indicated that the changes in the structural proteins (E, GP2, GP3, GP4, and GP5), and not the replicase, were responsible for the establishment of persistent infection in HeLa-L cell line by the HeLa-H-EAVP80 virus. It was further determined that recombinant viruses with substitutions in individual structural proteins were unable to establish persistent infection of the HeLa-L cells whereas a combination of substitutions in the E (Ser53→Cys, Val55→Ala), GP2 (Leu15→Ser, Trp31→Arg, Val87→Leu, and Ala112→Thr), GP3 (Ser115→Gly, Leu135→Pro), and GP4 (Tyr4→His, Ile109→Phe) proteins together, or a single point mutation in the GP5 protein (Pro98→Leu), created viruses that were able to establish persistent infection in HeLa-L cells (Fig. 5). In summary, these reverse genetic studies clearly showed that substitutions in the structural proteins rather than the replicase were responsible for establishment of persistent infection in HeLa-L cells by the HeLa-H-EAVP80 virus. Unlike the virulence determinants of EAV, the E and GP3 minor envelope proteins appeared to play an important role in the establishment of persistent infection in mammalian cells. In contrast, there were no amino acid substitutions in the unglycosylated M envelope protein suggesting that this protein has no role in the establishment of persistent EAV infection in mammalian cells.

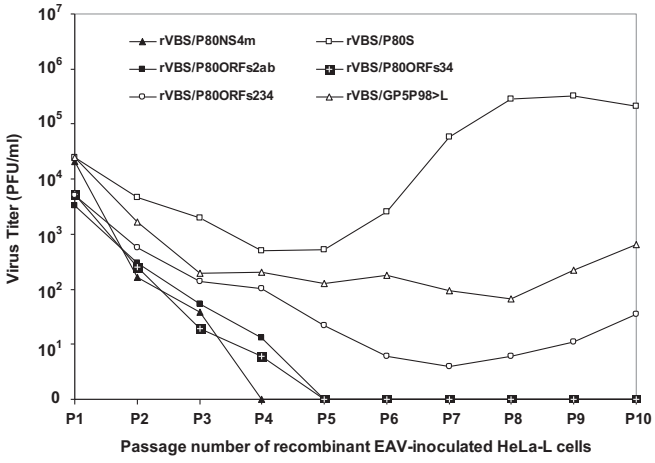

Fig. 5. Ability to establish persistent infection in HeLa-L cell line with recombinant viruses rVBS/P80NS4m, rVBS/P80S, rVBS/P80ORFs2ab, rVBS/P80ORFs34, rVBS/P80ORFs234, and rVBS/GP5P98→L. Tissue culture supernatants from serial subculture up to the 10th passage were harvested and titrated. The representative data of two separate experiments are shown. Adapted from Zhang et al. (2008c) with permission.

Characterization of viral determinants of tropism for CD3$^+$ T cells and CD14$^+$ monocytes

Recent studies in our laboratory have shown that the VBS virus not only can infect equine endothelial cells, CD14$^+$ monocytes, and lung macrophages, but also a small subpopulation of CD3$^+$ T cells (Go et al., 2010, 2011). In these studies, we evaluated the susceptibility of equine peripheral blood mononuclear cells (PBMCs) to infection with virulent (VBS) and attenuated (MLV) strains of EAV. Dual fluorescent antibody staining of PBMC cultures was performed using a panel of leukocyte differentiation antigen-specific monoclonal antibodies (MAbs) specific for pan CD3$^+$ T lymphocytes (CD4$^+$ helper T lymphocytes, CD8$^+$ cytotoxic T lymphocytes) and CD14$^+$ monocytes, as well as a MAb specific for EAV nsp1. The data suggested that the majority of CD3$^+$ T lymphocytes infected with the VBS virus were CD4$^+$ T lymphocytes rather than CD8$^+$ T lymphocytes. Furthermore, in contrast to the VBS virus, the MLV virus failed to infect CD3$^+$ T lymphocytes. We further investigated whether CD14$^+$ monocytes are equally susceptible to infection with the VBS and MLV viruses. Double-labeled flow cytometric analysis showed that monocytes could be infected with both virus strains. However, the mean percentage of cells infected with the MLV virus was significantly lower and remained near the lower limit of detection as compared to those detected in VBS virus-infected cultured equine monocytes. These findings confirm that not only CD3$^+$ T lymphocytes but also CD14$^+$ monocytes differ in their susceptibility to infection with the VBS and MLV strains of EAV.

Taken together, these data clearly suggested that the VBS and MLV vaccine strains of EAV differ in their ability to infect PBMCs. We therefore used two infectious cDNA clones, EAV rVBS, and EAV rMLV, as well as five chimeric viruses, rVBS/HK116 S (Zhang et al., 2008a), rVBS/MLV S, rMLV/VBS S, rMLV/VBS 234 and rMLV/VBS 56 (Go et al., 2010) (Fig. 6) to infect ex vivo preparations of equine PBMCs to identify the viral proteins involved in cellular tropism. The rVBS/HK116 S chimeric virus containing the structural proteins of the HK116 virus (which is fully attenuated for horses as compared to the VBS virus) in the rVBS backbone infected significantly fewer CD3$^+$ T lymphocytes as compared to the rVBS virus. In contrast, the percentage of CD14$^+$ monocytes infected by the two viruses was similar, indicating that the tropism of the HK116 strain had changed for CD3$^+$ T lymphocytes but not for CD14$^+$ monocytes following 116 passages in HK cells. When susceptible lymphocytes and monocytes were infected with rVBS/MLV S and rMLV/VBS S viruses, rVBS/MLV S did not infect CD3$^+$ T lymphocytes and replicated in CD14$^+$ monocytes at only a very low level, which was identical to what occurred following infection with the rMLV virus. In contrast, the rMLV/VBS S virus infected and replicated in both CD3$^+$ T lymphocytes and CD14$^+$ monocytes, similar to the rVBS virus. These results strongly suggest that the structural proteins of the VBS virus are responsible for determining its tropism for lymphocytes and monocytes. Furthermore, comparison of dual-color flow cytometric analysis of PBMCs infected with rVBS/HK116 S and rVBS/MLV S showed significant differences in CD14$^+$ monocyte infectivity, indicating that amino acid substitutions that occurred during further cell culture passage of the HK116 virus may have contributed to the change in monocyte tropism. In summary, these data suggest that viral tropism for CD3$^+$ T lymphocytes and CD14$^+$ monocytes was altered by amino acid changes in the envelope proteins of EAV (Table 6).

To evaluate the role of the minor and major envelope proteins in cellular tropism of EAV, additional recombinant viruses, rMLV/VBS 234 and rMLV/VBS 56, were generated using rMLV as the viral backbone. The rMLV/VBS 234 virus has a genome sequence identical to that of the rMLV virus except that the ORFs 2a, 2b, 3, and 4 (encoding the E, GP2, GP3, and GP4 minor envelope proteins) were replaced by the corresponding regions of the rVBS virus; the ORFs 5 and 6 (encoding the GP5 and M major envelope proteins) of the recombinant rMLV/VBS 56 virus were substituted with the corresponding genes of rVBS in the rMLV backbone. Unexpectedly, neither rMLV/VBS 234 nor rMLV/VBS 56 chimeras infected equine T lymphocytes (Fig. 6, panel k [rMLV/VBS 234] and panel m [rMLV/VBS 56]). However,

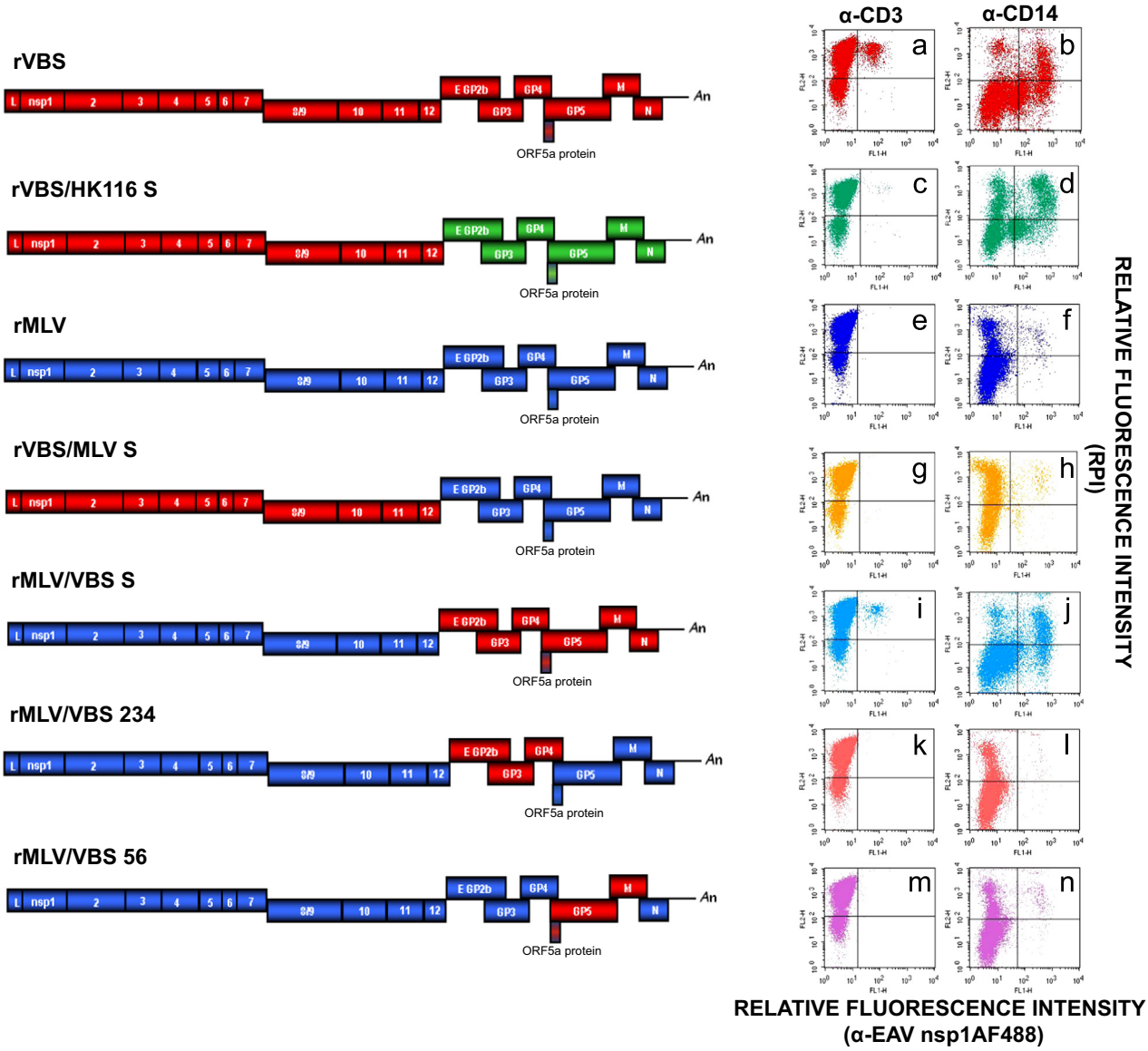

Fig. 6. Infection of lymphocytes and monocytes with chimeric EAV viruses. The genome of the infectious full-length cDNA clone of rVBS (red boxes) and the genome of the rMLV clone (blue boxes) are depicted. The genes encoding structural proteins of EAV HK116 virus are shown in green. The four chimeric viruses containing nonstructural and structural protein genes of either rVBS, or rMLV virus are also depicted. L, leader; *An*, poly A tail. The $CD3^+$ T lymphocytes and $CD14^+$ monocytes infected with recombinant viruses rVBS (panels a and b), rVBS/HK116 S (panels c and d), rMLV (panels e and f), rVBS/MLV S (panels g and h), rMLV/VBS S (panels i and j), rMLV/VBS 234 (panels k and l) and rMLV/VBS 56 (panels m and n) were examined by dual-color immunofluorescence flow cytometric analysis using MAbs against EAV nsp1 (12A4) and MAbs for cell specific cell surface antigens at 24 hours post infection (hpi). Adapted from Go et al. (2010) with permission.

comparisons in cultured monocytes showed that whereas the percentage of cells infected with rMLV/VBS 56 virus was similar to that of the rMLV and rVBS/MLV S viruses, infection rates for rMLV/VBS 234 virus were significantly lower (Fig. 6; panels n and l, respectively). Therefore, the higher relative fluorescence intensity values observed in monocytes infected with rMLV/VBS 56 (Fig. 6, panel n) as compared to rMLV/VBS 234 (Fig. 6, panel l), suggests that the GP5 and M protein sequences may play a greater role than those of E, GP2, GP3, and GP4 in facilitating monocyte infections, although both the major and minor envelope glycoproteins appear to be important in determining monocyte tropism. In summary, the data suggest that the difference in cellular tropism and virulence phenotype of the VBS and MLV strains of EAV is associated with the collective interactions of both major (GP5 and M) and minor (GP2, GP3, and GP4) envelope proteins. Furthermore, this study also demonstrated that $CD3^+$ T lymphocyte tropism is primarily determined by specific amino acid residues in the GP2, GP4, GP5, and M envelope proteins but not the GP3 minor envelope protein.

Characterization of viral determinants of mammalian cell tropism

In horses, EAV replicates in endothelial cells, blood mononuclear cells, selected epithelial cells, and myocytes (Balasuriya and Snijder, 2008; Del Piero, 2000; MacLachlan et al., 1996). Similarly, EAV replicates in a variety of primary cell cultures including equine pulmonary artery endothelial (Hedges et al., 2001), horse kidney, rabbit kidney, and hamster kidney cells, and a number of continuous cell lines including baby hamster kidney (BHK-21) (Hyllseth, 1969; Maess et al., 1970), rabbit kidney-13 (RK-13), African green monkey kidney (VERO) (Konishi et al., 1975; Radwan and Burger, 1973), rhesus monkey kidney (LLC-MK2),

Table 6
Amino acid substitutions between the EAV rVBS virus and the highly cell culture passaged EAV HK116 and ARVAC® MLV strains.

Open Reading Frames (ORFs)	Protein (aa length)	Amino acid differences[a]			
		Position	EAV rVBS (DQ846751)	EAV HK116 (EU586274)	ARVAC® (EU586275)
ORF2b (9824–10507)	**GP2** (227)	**62**	Tyr	Tyr	**His**
		92	Ile	**Thr**	**Thr**
		158	Gly	Gly	**Glu**
		223	Arg	**Pro**	**Pro**
ORF3 (10306–10797)[b]	**GP3** (163)[b]	**80**	Leu	Leu	**Val**
		123	Leu	Leu	**Ser**
		160	Cys	Cys	**Tyr**
		164	Stop	Stop	**Gln**
		165			**Phe**
		166			**Tyr**
		167			**Leu**
		168			**His**
		169			**Stop**
ORF4 (10700–11158)	**GP4** (152)	**4**	Tyr	Tyr	**Ser**
		8	Leu	**Ser**	**Ser**
		29	Ala	Ala	**Thr**
		37	Ile	Ile	**Thr**
ORF5 (11146–11913)	**GP5** (255)	**69**	Leu	Leu	**Pro**
		72	Gln	Gln	**Lys**
		81	Asn	**Asp**	**Asp**
		100	Ser	**Gly**	**Gly**
		104	Asn	**Gly**	**Gly**
		170	Ala	Ala	**Ser**
		214	Gly	Gly	**Glu**
ORF6 (11901–12389)	**M** (162)	**38**	Leu	Leu	**Ser**
		49	Phe	Phe	**Leu**
		71	Val	Val	**Ala**
		81	Met	**Thr**	**Thr**
		122	Ile	**Val**	**Val**
		150	Phe	**Phe**	**Cys**
		154	Ala	**Thr**	**Met**

Critical amino acid changes are identified in bold. All these changes are tested/confirmed by reverse genetics. Other major amino acid changes are identified in color.
[a] Amino acid substitutions are numbered according to the rVBS (GenBank accession number DQ846751).
[b] In ARVAC® MLV vaccine strain, the ORF3 spans from nucleotides 10,306–10,812 and the GP3 is 168 amino acids in length.

MARC-145, and hamster lung (HmLu) (Konishi et al., 1975) cells. In distinct contrast, PRRSV replicates in only a limited number of cell types that include primary porcine alveolar macrophages (PAM) and the African green monkey cell line, MA-104, or its derivative, CL2621, and MARC-145 (Van Breedam et al., 2010). Until recently, the viral envelope protein(s) involved in virus attachment and entry of EAV and PRRSV were poorly characterized (Das et al., 2011; Tian et al., 2012). Dobbe et al. (2001) demonstrated that a recombinant chimeric strain of EAV (based on EAV 030 backbone) expressing the ectodomain of GP5 of PRRSV IAF-Klop strain (Pirzadeh et al., 1998) did not alter cellular tropism. More recently, we used the prMLVB infectious cDNA clone to further characterize the role of the two major envelope proteins (GP5 and M) in the cellular tropism of EAV. Specifically, the prMLVB infectious cDNA clone was used as the backbone to generate a panel of 3 recombinant chimeric viruses by replacing the N-terminal ectodomains of the EAV GP5 and M proteins with those of the IA-1107 strain of North American PRRSV (Lu et al., 2012). The N-terminal ectodomain (aa 1–114) of EAV GP5 was replaced with the PRRSV GP5 ectodomain (aa 1–64) to generate the prMLVB4/5 GP5ecto construct; the N-terminal ectodomain (aa 1–16) of EAV M protein was replaced with the PRRSV M protein N-terminal ectodomain (aa 1–17) to generate the prMLVB4/5/6 Mecto construct; and both the GP5 and M N-terminal ectodomains of EAV were replaced with the PRRSV N-terminal ectodomains to generate the rMLVB4/5/6 GP5&Mecto construct. The three recombinant chimeric viruses (GenBank accession numbers JQ844156, JQ844157 and JQ844158) infected only EAV susceptible cell lines but not PAM cells, confirming unambiguously that the ectodomains of GP5 and M are not the major determinants of cellular tropism and consistent with the recent finding that the minor envelope proteins are the critical proteins in mediating cellular tropism of PRRSV (Tian et al., 2012).

Characterization of neutralization determinants of EAV

The major neutralization determinants of EAV have been mapped to the GP5 major envelope glycoprotein (encoded by ORF5) (Balasuriya et al., 1993, 1995, 1997, 2004a, b; Deregt et al., 1994; Chirnside et al., 1995; Glaser et al., 1995, Weiland et al., 2000). The comparative phenotypic characterization of field and neutralization-resistant variant (escape mutant [EM]) strains of

Table 7
Comparison of virulence phenotype of parental (EAV rVBS) and recombinant viruses with critical amino acid substitutions in the nsps and structural proteins.

Recombinant virus	Virulence phenotype	Body temperature	Edema	Nasal discharge	Conjunctivitis	Hemorrahage	Hives
EAV rVBS[ab]	Moderate to severe disease	Fever (38.9–40.6 °C) in 4 horses for 5–6 days	Moderate to severe limb edema in 4 horses for 4–6 days	Serous nasal discharge in 4 horses for 1–9 days	Mild conjunctivitis in 4 horses for 1–5 days	Petechial and ecchymotic hemorrhages in the oral mucous membranes of 4 horses for 2–5 days	Urticarial-type rash (hives) on the neck, shoulder and along the back of 2 horses for 2–3 days
rVBS/Vacc NS4m virus[ac]	Mild disease	Fever (39–39.6 °C) in 2 horses for 2–3 days	Moderate to severe limb edema in 3 horses for 11–12 days	None of the horses had a nasal discharge	Mild conjunctivitis in 1 horse for 4 days	Petechial hemorrhages in the oral mucous membranes of 2 horses for 1 day	No hives in any horse
rVBS/HK116 S virus[ac]	Very mild transient disease	Fever (39.4 °C) in 1 horse for 2 days	No limb edema in any horse	Mild serous nasal discharge in 3 horses for 3 days	No conjunctivitis in any horse	None of the horses developed mucosal hemorrhages	No hives in any horse

[a] Each virus was inoculated into 4 horses.
[b] Balasuriya et al. (2007).
[c] Zhang et al., (2008a).

EAV using both neutralizing MAbs and EAV strain-specific polyclonal equine antisera showed that antigenic variation in the V1 region of the GP5 protein principally was responsible for differences in the neutralization phenotype of various field strains of EAV. Genotypic and phenotypic characterization of these EM and field strains of EAV identified both conformational and linear neutralization determinants that include amino acids 49 (site A), 61 (site B), 67–90 (site C), and 98 through 106 (site D) in the GP5 protein (Balasuriya et al., 1997; Zhang et al., 2008b). Subsequently, reverse genetic manipulation of an infectious cDNA clone further characterized the neutralization determinants in the GP5 envelope glycoprotein (Balasuriya et al., 2004a). A panel of recombinant viruses was used in these studies, including chimeric viruses that each contained the ORF5 of different laboratory, field, and vaccine strains of EAV, a chimeric virus containing the N-terminal ectodomain of GP5 of a European strain of PRRSV, and mutant viruses with single site-specific substitutions in their GP5 proteins. The neutralization phenotype of each recombinant chimeric/mutant strain of EAV was determined with EAV-specific MAbs and polyclonal equine antisera, and compared to that of their parental viruses from which the substituted ORF5 was derived. Substitution of individual amino acids within the GP5 ectodomain usually resulted in differences in the neutralization phenotype of the recombinant viruses that were analogous to differences in the neutralization phenotype of field strains of EAV. These studies unequivocally confirm that the GP5 ectodomain contains critical determinants of EAV neutralization, that individual neutralization sites (A–D) are conformationally interactive, and that interaction of GP5 with the unglycosylated membrane protein M is likely critical to expression of individual epitopes in neutralizing conformation. Interestingly, there is some overlap in neutralization and virulence determinants located in the GP5 protein of EAV (Table 4).

Infectious cDNA clones as potential vaccine vectors

Development of infectious cDNA clones of positive-stranded RNA viruses and contemporary molecular biology techniques offer a new approach toward rational vaccine design and construction, by engineering defined mutations and/or deletions into the virus genome to produce attenuation and to minimize the likelihood of reversion to virulence (de Vries et al., 2000, 2001). Obviously, the substitutions or deletions that are introduced into the cloned virus genome must not hinder the recombinant virus from inducing protective immunity in vaccinated animals. Castillo-Olivares et al. (2003) described the generation of a candidate live marker vaccine for EAV by deletion of the major neutralization domain (aa 66–112) in the GP5 protein. This recombinant (deletion mutant) virus replicated to normal titer in cell culture, but at a lower rate than parental virus. Furthermore, two ponies immunized with this deletion mutant virus remained asymptomatic, however the virus was recovered from nasal secretions and/or blood for up to 14 DPI. The immunized ponies developed only a weak neutralizing antibody response as determined by serum neutralization assay using a virus (LP3A+ or CVL) derived by sequential passage of the original VBS virus. In contrast, the virus neutralizing antibody response was markedly stronger when assayed by SN test using the mutant virus. The immunized ponies were protected against challenge with a virulent laboratory strain of EAV (LP3A+); in contrast to the non-immunized controls, nasal shedding of virus and viremia were both minimal and transient in vaccinates. The authors concluded that an immune effector mechanism other than virus neutralizing antibody must exert a critical role in protection of the vaccinated ponies, and they also showed that the vaccinated ponies readily could be distinguished from the ponies infected only with wild type virus using the GP5-peptide ELISA (GP5-OVA ELISA; aa 81–106) described by Nugent et al. (2000). Therefore, vaccination of horses with such a deletion mutant marker vaccine can potentially facilitate the serological discrimination between vaccinated and naturally infected horses (so-called DIVA strategy). Similarly, the EAV 030 cDNA clone has been used to develop disabled infectious single-cycle (DISC) mutants using complementing cell lines expressing minor structural proteins (GP2 [formally G_S], GP3, and GP4) (Zevenhoven-Dobbe et al., 2004). However, vaccines based on recombinant DNA technology have not yet been adopted for field use by the equine industry.

A stable full-length cDNA clone of a MLV vaccine strain of EAV has been recently developed (pEAVrMLV; Zhang et al., 2012), and the recombinant virus (rMLV) from it has 100% nucleotide identity to the parental MLV vaccine strain of EAV. A single silent nucleotide substitution was introduced into the nucleocapsid gene (pEAVrMLVB), enabling the cloned vaccine virus (rMLVB) to be distinguished from parental MLV vaccine as well as other field and laboratory strains of EAV using an allelic discrimination real-time RT-PCR assay. *In vivo* studies confirmed that the cloned vaccine virus was safe and induced high titers of neutralizing antibodies against EAV in experimentally immunized horses. However, when challenged with the heterologous EAV KY84 strain, the rMLVB vaccine virus protected immunized horses as reflected by reduced magnitude and duration of viremia and virus shedding, but

vaccination did not prevent development of signs of EVA, although these were reduced in clinical severity. While it is believed there is only one known serotype of EAV, field strains differ in their neutralization phenotype (Balasuriya et al., 1995, 1997, 2004a; MacLachlan and Balasuriya, 2006; Miszczak et al., 2012; Zhang et al., 2010). It has been shown previously that the serum from horses vaccinated with the MLV vaccine strain neutralizes some EAV field strains, such as KY84, to only a relatively low titer ($\leq$1:8–1:64) (Balasuriya et al., 1997, 2004a; Zhang et al., 2010), which may explain why the vaccinated horses were not completely protected against clinical signs of EVA following challenge with the heterologous EAV KY84 strain. This also further confirms the importance of high titer neutralizing antibodies ($\geq$1:64) in protecting against the clinical signs of EAV infection (Fukunaga and McCollum, 1977; Timoney et al., 1988). These recent data also emphasize the importance of conducting additional in-depth cross-neutralization, and perhaps vaccine challenge studies using more recent EAV isolates representing all three phylogenetic clades of EAV (North American and two European [EU-1 and EU-2]).

The infectious cDNA clone of the MLV vaccine strain of EAV provides a resource that could be used to design and to develop more broadly protective recombinant MLV vaccines by systematically incorporating key neutralization epitopes from various EAV isolates of significantly distinct neutralization phenotypes. Furthermore, the vaccine clone pEAVrMLVB could be further manipulated to improve the vaccine efficacy as well as to develop a marker vaccine for serological differentiation of EAV naturally infected from vaccinated animals (DIVA).

Lessons learned and insights gained

Development and reverse genetic manipulation of several infectious cDNA clones of well-characterized EAV strains have clearly shown that multiple viral genes are involved in determining virus phenotype (e.g. virulence/attenuation, viral persistence, cellular tropism, and neutralization). These studies confirm that amino acid substitutions in both structural and nonstructural proteins may allow field strains of EAV to evade protective host immune responses to facilitate persistent infection of stallions. Importantly, differences in viral phenotype are typically not associated with a single coding change in a specific viral gene that leads to single amino acid change, rather these differences in phenotype can involve multiple and different changes in viral genes (multigenic) that lead to emergence/selection of variants with diverse phenotypes. Although the use of infectious cDNA clones derived from viruses of defined phenotype has allowed us to better characterize the viral determinants of behavior, it is to be stressed that the nucleotide and amino acid changes identified in laboratory strains of EAV may not be identical and/or not contribute to the same phenotypic change in field strains of the virus that occur naturally. Furthermore, it is important to emphasize that we have not performed any detailed biochemical functional analyzes to identify the effect of each amino acid change in specific nsps that might be responsible for the attenuation/virulence phenotype of EAV. Therefore, extrapolation of findings from these laboratory studies to identification of genetic determinants of key phenotypic properties (e.g. virulence, persistence etc.) of field strains of EAV should be done cautiously. Lastly, it is to be stressed that identification of critical attenuating mutations within either nonstructural or structural viral proteins provides the opportunity to prospectively design a new, safe, and efficacious genetically engineered MLV vaccine against EVA.

In summary, selection and careful analysis of genetic and phenotypic data from well-characterized EAV strains, including their reverse genetic manipulation, has led to the identification of apparently critical determinants of phenotypic properties of the virus. Similar approaches using well characterized strains (and their respective cDNA clones) of other arteriviruses (PRRSV, LDV, SHFV, WPDV) will also facilitate studies to define the molecular basis of viral virulence, neutralization, persistence, and cellular tropism.

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