



Chimeric viruses containing the N-terminal ectodomains of GP5 and M proteins of porcine reproductive and respiratory syndrome virus do not change the cellular tropism of equine arteritis virus

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ABSTRACT

Equine arteritis virus (EAV) and porcine reproductive and respiratory syndrome virus (PRRSV) are members of family *Arteriviridae*; they are highly species specific and differ significantly in cellular tropism in cultured cells. In this study we examined the role of the two major envelope proteins (GP5 and M) of EAV and PRRSV in determining their cellular tropism. We generated three viable EAV/PRRSV chimeric viruses by swapping the N-terminal ectodomains of these two proteins from PRRSV IA1107 strain into an infectious cDNA clone of EAV (rMLVB4/5 GP5ecto, rMLVB4/5/6 Mecto and rMLVB4/5/6 GP5&Mecto). The three chimeric viruses could only infect EAV susceptible cell lines but not PRRSV susceptible cells in culture. Therefore, these data unequivocally demonstrate that the ectodomains of GP5 and M are not the major determinants of cellular tropism, further supporting the recent findings that the minor envelope proteins are the critical proteins in mediating cellular tropism (Tian et al., 2012).

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Introduction

Members of the family *Arteriviridae* include equine arteritis virus (EAV), porcine reproductive and respiratory syndrome virus (PRRSV), lactate dehydrogenase-elevating virus (LDV) of mice and simian hemorrhagic fever virus (SHFV) (Cavanagh, 1997; Faaberg et al., 2012). Arteriviruses are small, enveloped animal viruses with an isometric core containing a positive sense RNA genome (Snijder and Meulenberg, 1998, 2001). EAV and PRRSV are the causative agents of equine viral arteritis (EVA) in horses and porcine reproductive and respiratory syndrome (PRRS) in pigs, respectively (Doll

et al., 1957; Russell et al., 1980; Wensvoort et al., 1991). The EAV and PRRSV genomes are polycistronic and contain at least 10 open reading frames [ORFs; (den Boon et al., 1991; Firth et al., 2011; Johnson et al., 2011)]. The 5'-proximal three-quarters are occupied by two large ORFs (1a and 1b) that together encode all viral enzyme functions (collectively referred to as "replicase") required for genome replication and subgenomic mRNA (sgmRNA) production (Fang and Snijder, 2010; Snijder and Spaan, 2006). The ORFs 2a, 2b, 3, 4, 5a, 5, 6, and 7 of EAV and PRRSV are partially overlapping and are located in the 3'-one-quarter of the genome, encoding seven envelope proteins [E, GP2, GP3, GP4, ORF5a protein, GP5 and membrane (M)] and a nucleocapsid protein (N) (Firth et al., 2011; Johnson et al., 2011; Snijder and Meulenberg, 1998, 2001; Ziebuhr et al., 2000). The glycosylated GP5 and the unglycosylated M protein (encoded by ORF5 and ORF6, respectively) are the major envelope proteins of EAV and PRRSV; they form a disulfide-linked heterodimer in the mature virus particles (de Vries et al., 1995; Mardassi et al., 1996). The formation of GP5/M heterodimer is critical for the expression of neutralization epitopes of both viruses (Balasuriya et al., 2002, 2000; Jiang et al., 2006; Ostrowski et al., 2002). The M protein is the most conserved envelope protein of arteriviruses (Faaberg, 2008). The GP5/M heterodimer is presumed to play important roles in attachment to the host cell receptor and in cell entry (Snijder and Meulenberg, 1998). In addition, the arterivirus

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envelope contains a heterotrimer of three minor membrane glycoproteins (GP2 [encoded by ORF2b in EAV and ORF2a in PRRSV], GP3 [encoded by ORF3] and GP4 [encoded by ORF4]) and two unglycosylated envelope proteins (E [ORF2a in EAV and ORF2b in PRRSV] (Wu et al., 2001, 2005) and ORF5a protein [ORF5a]) (Firth et al., 2011; Johnson et al., 2011). It has also been shown by reverse genetics that the elimination of ORF5a protein expression by knocking out the start and stop codon will cripple EAV virus and lead to progeny virus with a small plaque phenotype and a significantly reduced virus titer in transfected cells (Firth et al., 2011).

Arteriviruses are highly species specific and macrophages are the primary target cells of virus replication (Balasuriya and Snijder, 2008; Duan et al., 1997; Kim et al., 1993; Lawson et al., 1997; Plagemann and Moennig, 1992; Rossow, 1998). However, EAV can replicate in a variety of cells 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), MARC-145 and hamster lung (HmLu) (Konishi et al., 1975) cells. In contrast, PRRSV can only replicate in a limited number of cell types. North American PRRSV (Type 2) strains replicate in primary porcine alveolar macrophages (PAM), and the African green monkey cell line, MA-104, or its derivative, MARC-145 (Van Breedam et al., 2010a). Most, if not all, European PRRSV (Type 1) strains replicate best or exclusively in PAMs, but can be adapted to grow in MA-104 derived cell lines, including CL2621 and MARC-145. Until

recently, the viral envelope protein(s) involved in virus attachment and entry of EAV and PRRSV have not been fully characterized (Das et al., 2011; Tian et al., 2012). In a previous study, Dobbe et al. (2001) demonstrated that EAV expressing the ectodomain of GP5 of PRRSV IAF-Klop strain (Pirzadeh et al., 1998) did not change the cellular tropism of the virus. Recently, in our laboratory, we have developed an infectious cDNA clone of the modified live virus (MLV) vaccine (ARVAC[®]) of EAV (prMLVB) (Zhang et al., submitted for publication). This infectious cDNA clone was originally developed to design a marker vaccine for EAV and to increase the safety and efficacy of the current MLV vaccine, as well as a vector platform to express heterologous genes from other viruses. In this study, we describe the use of this infectious cDNA clone to characterize the role of the major envelope proteins (GP5 and M) for investigating the cellular tropism of EAV.

Results and discussion

Generation of EAV/PRRSV chimeric viruses

The infectious cDNA clone (prMLVB) of the MLV vaccine strain of EAV (ARVAC[®]) was used as the backbone to generate a panel of 5 recombinant chimeric viruses by replacing either the full-length or the N-terminal ectodomains of GP5 and M proteins of EAV with the IA-1107 strain of North American PRRSV (Fig. 1, Table 1). Because of the overlapping gene arrangement in the 3' end of the EAV genome, it was necessary to separate ORF 5 from flanking ORFs 4 and 6 without disrupting the coding sequences of each of

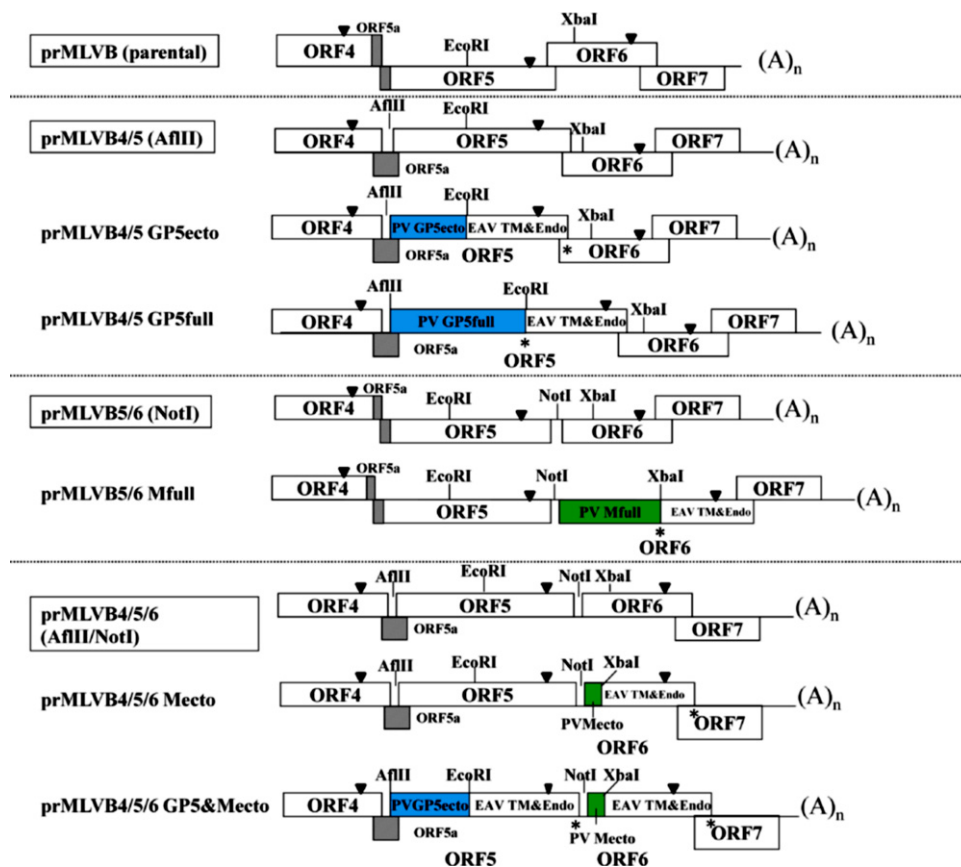


Fig. 1. Schematic presentation of parental, recombinant and chimeric cDNA clones. Only the ORFs 4 to 7 and 3' nontranslated region is depicted. The restriction sites introduced to separate ORF4 and 5 (AflII), and ORF 5 and 6 (NotI), and ORF 4, 5, and 6 (AflII and NotI) are depicted. The transcriptional regulatory sequences (TRS) that regulate leader-body junction during viral subgenomic RNA synthesis are indicated by triangles. The stop codon for each chimera is identified with an asterisk (*). The N-terminal ectodomain or full-length of PRRSV GP5 protein is labeled in blue. The N-terminal ectodomain or full-length of PRRSV M protein is labeled in green.

Table 1
Composition of the GP5 and M proteins of EAV–PRRSV chimeric constructs.

Construct	Chimeric protein	GP5	Fused to	M	Fused to	Production of progeny virus ^a	Plaque size ^b (mm)	GenBank number
prMLVB	n.a. ^c	Parental (pt) EAV GP5	n.a.	pt EAV M	n.a.	+	5	FJ798196
prMLVB4/5	n.a.	pt EAV GP5	n.a.	pt EAV M	n.a.	+	2.4	JQ844153
prMLVB4/5 GP5ecto	GP5ecto	PRRSV GP5 ectodomain a.a. 1–64	EAV GP5 a.a. 115–255	pt EAV M	n.a.	+	1	JQ844156
prMLVB4/5 GP5full	GP5full	PRRSV GP5 full-length a.a. 1–200	n.a.	pt EAV M	n.a.	–	n.a.	n.a.
prMLVB5/6	n.a.	pt EAV GP5	n.a.	pt EAV M	n.a.	+	3	JQ844154
prMLVB5/6 Mfull	Mfull	pt EAV GP5	n.a.	PRRSV M full-length a.a. 1–175	n.a.	–	n.a.	n.a.
prMLVB4/5/6	n.a.	pt EAV GP5	n.a.	pt EAV M	n.a.	+	2.8	JQ844155
prMLVB4/5/6 Mecto	Mecto	pt EAV GP5	n.a.	PRRSV M ectodomain a.a. 1–17	EAV M a.a. 17–162	+	0.5	JQ844157
prMLVB4/5/6 GP5&Mecto	GP5&Mecto	PRRSV GP5 ectodomain a.a. 1–64	EAV GP5 a.a. 115–255	PRRSV M ectodomain a.a. 1–17	EAV M a.a. 17–162	+	0.5	JQ844158

^a The production of progeny virus was evaluated by IFA staining with EAV N antibody and development of cytopathic effect.

^b Plaque morphology study was done in EEC.

^c Not applicable.

the three ORFs. The ORFs 4 and 5, ORFs 5 and 6, and ORFs 4, 5 and 6 were separated in the prMLVB plasmid to generate the plasmids prMLVB4/5, prMLVB5/6 and prMLVB4/5/6 (GenBank accession numbers JQ844153, JQ844154 and JQ844155), respectively. The authenticities of these plasmid constructs were first confirmed by sequencing ORFs 4–7 (data not shown). The same amount of full-length *in vitro* transcribed (IVT) RNA generated from linearized prMLVB, as well as mutant prMLVB4/5, prMLVB5/6 and prMLVB4/5/6 cDNA clones were electroporated into BHK-21 cells for generation of recombinant viruses as previously described (Balasuriya et al., 1999; van Dinten et al., 1997). The viability of each recombinant clone was determined by indirect immunofluorescence assay (IFA) using EAV N protein specific monoclonal antibody (MAb 3E2) to stain BHK-21 cells 24 h post transfection with IVT RNA generated from each linearized plasmid construct (data not shown). The cells transfected with each of the three recombinant transcripts produced 90–100% cytopathic effect (CPE) in transfected cells after 72–96 h post transfection, and virus stocks were made from these tissue culture fluids (TCF; P0). The authenticity of all three recombinant viruses was confirmed by sequencing ORFs 4–7 from RNA extracted from each TCF (P0). All virus sequences were identical to the plasmid sequence from which they were derived. The TCFs (P0) containing each recombinant virus were then used to infect BHK-21 cells at a multiplicity of infection (MOI) of 1 and progeny viruses were collected at different time points for titration. The growth characteristics and plaque morphology were compared in RK-13 and EEC, respectively. All of the recombinant rMLVB4/5, rMLVB5/6 and rMLVB4/5/6 viruses had the highest progeny virus titer at 48 h post infection. They had approximately 2 log₁₀ lower titer as compared to the parental rMLVB virus at this time point (Fig. 2). When compared to the parental virus, the recombinant viruses showed a 48–60% reduction in plaque size in EECs. The parental rMLVB produced 5 mm diameter large sized plaques while the recombinant rMLVB4/5, rMLVB5/6 and rMLVB4/5/6 produced 2.4, 3 and 2.8 mm diameter medium sized plaques in EECs, respectively. However, there was no significant difference in growth characteristics among these three recombinant viruses. All three recombinant viruses were stable for more than 10 serial passages in BHK-21 cells. The separation of these ORFs did not alter the amino acid sequence of GP4, GP5 and M proteins of EAV but the ORFs 4/5 separation changed the N-terminal amino acid sequence of ORF5a protein (Fig. 3). In a recent study, we demonstrated that complete

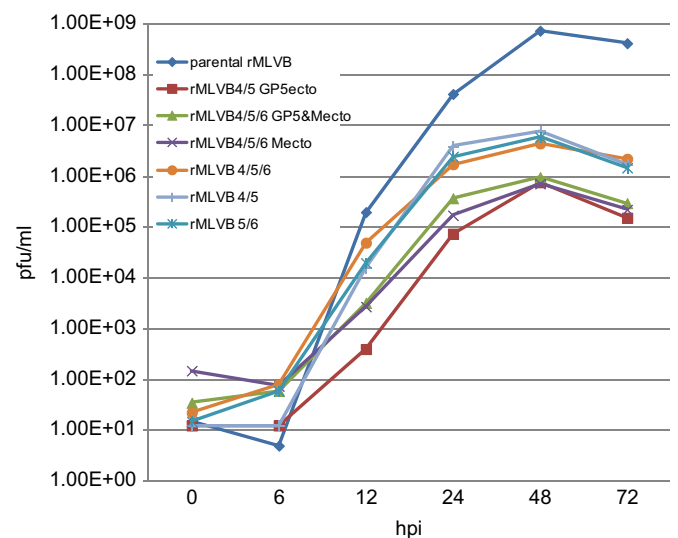


Fig. 2. Replication characteristics of parental rMLVB, recombinant rMLVB4/5, rMLVB5/6 and rMLVB4/5/6 and chimeric rMLVB4/5 GP5ecto, rMLVB4/5/6 Mecto and rMLVB4/5/6 GP5&Mecto viruses in BHK-21 cells. Subconfluent monolayers of BHK-21 cells were grown in 6-well plates, inoculated with each P0 virus (MOI of 1) and incubated at 37 °C for 1 h. After removal of the inoculum, the cells were rinsed three times with PBS and overlaid with 4 ml of EMEM. At indicated time points, supernatants were harvested and titrated by plaque assay in RK-13 cells.

elimination of ORF5a protein experimentally could lead to 2–3 log lower virus titer and tiny plaques (Firth et al., 2011). Interestingly, the addition of extra nucleotides to the ORF5a coding sequence did not cripple the recombinant viruses to the same extent as the ORF5a knockout virus (Firth et al., 2011) indicating that this region of the EAV genome could accept insertion of a small stretch of nucleotides (see below). Collectively, these data demonstrated that separation of these ORFs have somewhat compromised the derived viruses but the recombinant constructs were stable and could be used for further studies. The next step was to generate a panel of EAV–PRRSV chimeric plasmids by replacing the nucleotide (nt) sequence encoding the N-terminal ectodomain or full-length of GP5 and/or the M protein on an EAV backbone with corresponding sequences from North American PRRSV IA1107 strain using prMLVB4/5, prMLVB5/6 and prMLVB4/5/6 plasmids (Fig. 1, Table 1). We have exchanged the N-terminal ectodomain

Parental virus

EAV rMLVB ORF5a 1 12 15 59
 MFFYDWYVGLN**NAIY**DCIVILALGCAITCLLLILHRSALHRLHLVHADGSRRYCQFAAI

EAV rMLVB ORF5 1 48>>>116
 MLSMIVLLFLLWGAPSHAYFSYYTAQRFTDFTLCMLTDRGVIANLLRY>>>>

EAVrMLVB ORF 4/5 separation

EAV rMLVB4/5 ORF5a 1 12 23
 MFFYDWYVGLN**SAICELKQH**AVHDCIVILALGCAITCLLLILHRSALHRLHLVHADGSRRYCQFAAI

EAV rMLVB4/5 ORF5 1 109 115 134 255
 MLSMIVLLF.....FIYYGR**EGFIVVLDVFMFYPVLVLF**.....

PRRSV IA1107 strain

PRRSV IA1107 ORF5a 6 46
 MFRYV**GEM**LD**RGLLLAVAFFV**YRA**VLFC**AR**QRQQQQLPFTV**NL

PRRSV IA1107 ORF5 64 200
MLGKCLTAGCCSQLPFLWCIVPFCAALVNASSSSSSQLQSIYNLTICELNGTDWL**NKNFDWAV**ETVIFPV>>>>

EAV-PRRSV GP5 N-terminal ectodomain chimera

GP5ecto chimera virus ORF5a 12 21 62
 MFFYDWYVGLN**SAICELKQHV**GEMLD**RGLLLAVAFFV**YRA**VLFC**AR**QRQQQQLPFTV**NL

GP5ecto chimera virus ORF5 64 65 205
MLGKCLTAGCCSQLPFLWCIVPFCAALVNASSSSSSQLQSIYNLTICELNGTDWL**NKNFDWAV****EGFIVVLDVFMFYPVLVLF**.....

Fig. 3. Sequence alignments of amino acids of the ORF5a and GP5 proteins of parental EAV and chimeric viruses. The amino acid sequence changes due to ORF4 and 5 separations are highlighted in blue. The remaining ORF5 from EAVrMLVB backbone in the GP5ecto chimera construct is marked in green. The ectodomains of PRRSV are labeled in red. The common region of ORF5a between PRRSV and GP5ecto chimera is labeled in purple. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(a.a. 1–64) and full-length (a.a. 1–200) of PRRSV GP5 with ectodomain (a.a. 1–114) of EAV GP5 to generate prMLVB4/5 GP5ecto and prMLVB4/5 GP5full constructs on a prMLVB4/5 backbone. Similarly, the N-terminal ectodomain (a.a. 1–17) and full-length (a.a. 1–175) of PRRSV M were exchanged with the N-terminal ectodomain (a.a. 1–16) of EAV M protein, respectively, to generate prMLVB4/5/6 Mecto and prMLVB5/6 Mfull constructs on the prMLVB4/5/6 and prMLVB5/6 backbones, respectively (Fig. 1, Table 1). The ORF5 and ORF6 regions of all chimeric plasmid constructs were RT-PCR amplified with two EAV specific primers (10763P and 12568N) and visualized by agarose gel electrophoresis (data not shown). Subsequently, the RT-PCR products were sequenced to confirm the authenticity of the chimeric plasmid constructs. All chimeric constructs maintained in-frame coding sequences in ORF5a, ORF5 and ORF6. Full-length IVT RNAs from linearized chimeric plasmids were transfected into BHK-21 cells as previously described (Balasuriya et al., 1999; van Dinten et al., 1997). A portion of transfected cells were seeded into chamber slides for IFA staining to check for the expression of EAV N protein 24 h post transfection. With the exception of BHK-21 transfected with prMLVB4/5 GP5full and prMLVB5/6 Mfull, cells transfected with prMLVB4/5 GP5ecto, prMLVB4/5/6 Mecto and prMLVB4/5/6 GP5&Mecto stained positive for EAV N protein (data not shown). This observation was further confirmed by the development of 90–100% CPE 72–120 h post transfection in BHK-21 cells that were transfected with IVT RNA from prMLVB4/5 GP5ecto, prMLVB4/5/6 Mecto and prMLVB4/5/6 GP5&Mecto. The experiments have been repeated twice on separate occasions. Collectively, these data suggested that only 3 of the 5 chimeric constructs could produce infectious progeny viruses. Each of the three progeny viruses was harvested (P0) and subjected to further *in vitro* characterization. Of the 5 chimeric constructs, only rMLVB4/5 GP5ecto, rMLVB4/5/6 Mecto and rMLVB4/5/6 GP5&Mecto could

generate infectious progeny viruses following transfection of IVT RNA into BHK-21 cells (GenBank accession numbers JQ844156, JQ844157 and JQ844158, respectively). The IVT RNA generated from plasmids containing full-length GP5 or M replacement of PRRSV IA-1107 strain (prMLVB4/5 GP5full and prMLVB5/6 Mfull) failed to generate infectious progeny viruses. The genetic stabilities of each of the three replicating chimeric viruses were determined by 10 serial passages in BHK-21 cells and subsequent RT-PCR amplification and sequencing of ORF5–6 from RNA extracted from P0, P5 and P10 TCF. The sequence data revealed no substitutions, insertions or deletions in this region of all three chimeric viruses following serial cell culture passage. These results unequivocally demonstrated that these three chimeric viruses are highly stable following serial passage in cell culture. The failure to generate chimeric viruses containing full-length PRRSV GP5 or M protein on the EAV backbone may be due to the fact that being the smallest arterivirus in the family *Arteriviridae*, EAV has evolved to the point that it is unable to accept any large nucleotide inserts into the genome. However, it has been reported that EAV can tolerate small segments of nucleotides in the structural proteins genes (de Vries et al., 2001; Dobbe et al., 2001). It is also possible that the conformational interaction between GP5 and M proteins requires both the homologous transmembrane and cytoplasmic tails of these two proteins for proper protein folding and expression. We cannot exclude other possibilities such as the cytoplasmic domains of the GP5 and M proteins are required for specific interaction with other viral proteins such as nucleocapsid protein for the assembly of infectious virions.

Growth characteristic and plaque morphology of EAV/PRRSV chimeric viruses

The growth characteristics and plaque morphologies of rMLVB4/5 GP5ecto, rMLVB4/5/6 Mecto and rMLVB4/5/6 GP5&Mecto

chimeric viruses were compared in RK-13 cells and EEC, respectively, as described before. All three EAV–PRRSV chimeric viruses rMLVB4/5 GP5ecto, rMLVB4/5/6 Mecto and rMLVB4/5/6 GP5&Mecto had similar growth curves. Replication titers were 3 log₁₀ lower as compared to parental rMLVB virus and 1 log lower as compared to the recombinant cloned viruses (rMLVB 4/5 and rMLVB4/5/6); these were derived 48 h post infection (Fig. 2). Furthermore, P5 and P10 TCF of all three chimeric viruses had similar growth curves as compared to P0 TCF (Table 2). Plaque assays were performed in EECs for all three chimeric viruses and their plaque morphology was compared to parental rMLVB, recombinant rMLVB4/5, rMLVB5/6 and rMLVB4/5/6 viruses. The three chimeric viruses (rMLVB4/5 GP5ecto, rMLVB4/5/6 Mecto and rMLVB4/5/6 GP5&Mecto) produced small sized plaques (0.5 mm to 1 mm) as compared to the parental rMLVB virus (5 mm) and the three recombinant viruses from which the chimeric viruses were derived from 2.4–3 mm). The lower titers and smaller plaque sizes of the chimeric viruses clearly indicated that although the viruses are genetically stable (Fig. 4), they have significantly compromised phenotypes as reflected in limited replication and spreading when compared to the parental rMLVB or the three recombinant viruses from which they were derived (ORF4/5, ORF5/6 and ORFs4/5/6). Interestingly, when progeny viruses (P0) produced in BHK-21 cells were titrated in RK-13 cells for growth curve analysis, the reduction in titer of the chimeric viruses was not as significant as that observed in EECs, in which there was a reduction in plaque size (medium to pinpoint). This observation suggested that infection and spreading was influenced by the cell type in which the viruses are propagated. Specifically, EAV field strains produced different plaque sizes in EEC but not in RK-13 (Moore et al., 2003). Taken together, the data clearly showed that the presence of PRRSV N-terminal ectodomains of GP5 and M altered the growth characteristics and cell to cell spread of the chimeric viruses. Furthermore, it has long been proposed that the GP5 and M proteins of arteriviruses play a major role in virus attachment (Balasuriya et al., 1997; Van Breedam et al., 2010b). Similarly, nonspecific interactions have been shown between PRRSV M protein and a heparin-like receptor on PAM cells (Delputte et al., 2002).

Cellular tropism of EAV/PRRSV chimeric viruses

Although EAV and PRRSV infect cells from the monocyte/macrophage lineage in their natural host, they present different cellular tropisms in cell culture (Duan et al., 1997; Plagemann and

Moennig, 1992; Snijder and Meulenbergh, 1998). EAV can infect a wide variety of cell lines while PRRSV can only infect PAM and African green monkey kidney (MA-104) cells and the derivative (MARC-145) cell line (Kim et al., 1993; Snijder and Meulenbergh, 1998). The chimeric viruses derived by swapping the ectodomains of PRRSV GP5 and M proteins into an EAV backbone provide valuable tools to study cellular tropisms. The three viable chimeric viruses were tested in different EAV-susceptible cell lines (EEC, RK-13, BHK-21, MARC-145), PRRSV-susceptible cells (PAM and MARC-145) and porcine alveolar macrophage cell line (3D4/21) to confirm cellular tropism and infectivity of the progeny virus. Virus replication in various cell lines was determined by IFA staining with MAb 3E2 (α -N of EAV) and polyclonal pig anti-PRRSV serum (Fig. 5 and Table 3). Interestingly, the three chimeric viruses (rMLVB4/5 GP5ecto, rMLVB4/5/6 Mecto and rMLVB4/5/6 GP5&Mecto), along with the rMLVB parental virus, were able to infect EEC, MARC-145, BHK-21, and RK-13 cells but not PAM and 3D4/21 cells as determined by IFA staining for the N protein of EAV (Fig. 5). PRRSV was able to infect PAM and MARC-145 cells but not EEC, BHK-21, RK-13 and 3D4/21 cells based on presence or absence of staining with PRRSV-specific monoclonal antibody against the nucleocapsid protein (Fig. 5). Furthermore, MARC-145 cells were infected with the wild type PRRSV, parental rMLVB and the three chimeric viruses (rMLVB4/5 GP5ecto, rMLVB4/5/6 Mecto and rMLVB4/5/6 GP5&Mecto) on IFA staining with pig anti-PRRSV polyclonal serum. Positive staining was observed in MARC-145 cells infected with PRRSV and three chimeric viruses indicating that PRRSV GP5 and/or M proteins are expressed in the three chimeric viruses (Fig. 5B). None of the viruses were able to infect 3D4/21 cells. This finding was consistent with previous observations that 3D4/21, a continuous porcine alveolar macrophage cell line, does not support replication of PRRSV (Weingartl et al., 2002). The exchange of GP5 and/or M protein N-terminal ectodomains from PRRSV (individual or double) could not alter the tropism of the chimeric viruses. Similarly, a previous study has shown that exchanging the GP5 ectodomains from another PRRSV IAF-Klop strain did not change the cell tropism of EAV or PRRSV (Dobbe et al., 2001). In that study, it was shown that chimeric viruses containing the N-terminal ectodomains of M envelope proteins of PRRSV and LDV on an EAV backbone are replication- and transcription-competent (as determined by the synthesis of both non-structural and structural proteins), but unable to produce infectious progeny virus. Dobbe et al. (2001) indicated that this was due to the failure of formation of the GP5/M heterodimer in the chimeric proteins. In contrast, we demonstrated that the N-terminal ectodomain of the M protein of EAV can be replaced by the N-terminal ectodomain of PRRSV M protein without compromising the production of viable progeny virus using a different infectious cDNA clone of EAV and a more recent North American strain of PRRSV IA1107. Our findings indicate that neither the GP5 nor M protein is involved in determining the cellular tropism of EAV. This supports the hypothesis that EAV and perhaps PRRSV major envelope proteins are not determinants of the cellular tropism of these two viruses. Interestingly, in a very recent study, a PRRSV infectious cDNA clone was used as a vector to construct a chimera in which PRRSV ORFs 2a, 2b, 3 and 4 were replaced by the corresponding ORFs from EAV (Tian et al., 2012). This PRRSV/EAV chimeric virus acquired the broad cell tropism that is typical of EAV, which clearly indicated that the minor envelope protein (GP2, GP3 and GP4) and E protein play a key role in determining the cellular tropism of arteriviruses and these play a major role in virus attachment and entry (Tian et al., 2012). However, the major neutralization determinants of EAV and PRRSV are both localized in the N-terminal ectodomain of GP5 (Balasuriya et al., 1995, 1997; Ostrowski et al., 2002; Plagemann, 2004; Plagemann et al.,

Table 2
Stability of EAV–PRRSV chimeric viruses following 10 serial passages in BHK-21 cells.

Virus passage	Virus titer (pfu/ml)		
	rMLVB4/5 GP5ecto	rMLVB4/5/6 Mecto	rMLVB4/5/6 GP5&Mecto
P0 ^a	3.5×10^6	8.2×10^5	2.6×10^6
P1	1.1×10^6	3.8×10^5	3.6×10^6
P2	1.2×10^6	3.4×10^5	1.5×10^6
P3	1.3×10^6	5.1×10^5	2.1×10^6
P4	8.3×10^5	2.6×10^5	2.4×10^6
P5 ^a	1.0×10^6	2.5×10^5	2.5×10^6
P6	5.5×10^6	8.9×10^5	3.0×10^6
P7	1.3×10^6	1.6×10^5	1.3×10^6
P8	5.9×10^6	2.6×10^5	5.4×10^6
P9	2.0×10^6	1.8×10^5	1.7×10^6
P10 ^a	9.7×10^6	7.3×10^5	2.3×10^6

^a P0, P5 and P10 viruses were sequenced and found genetically stable.

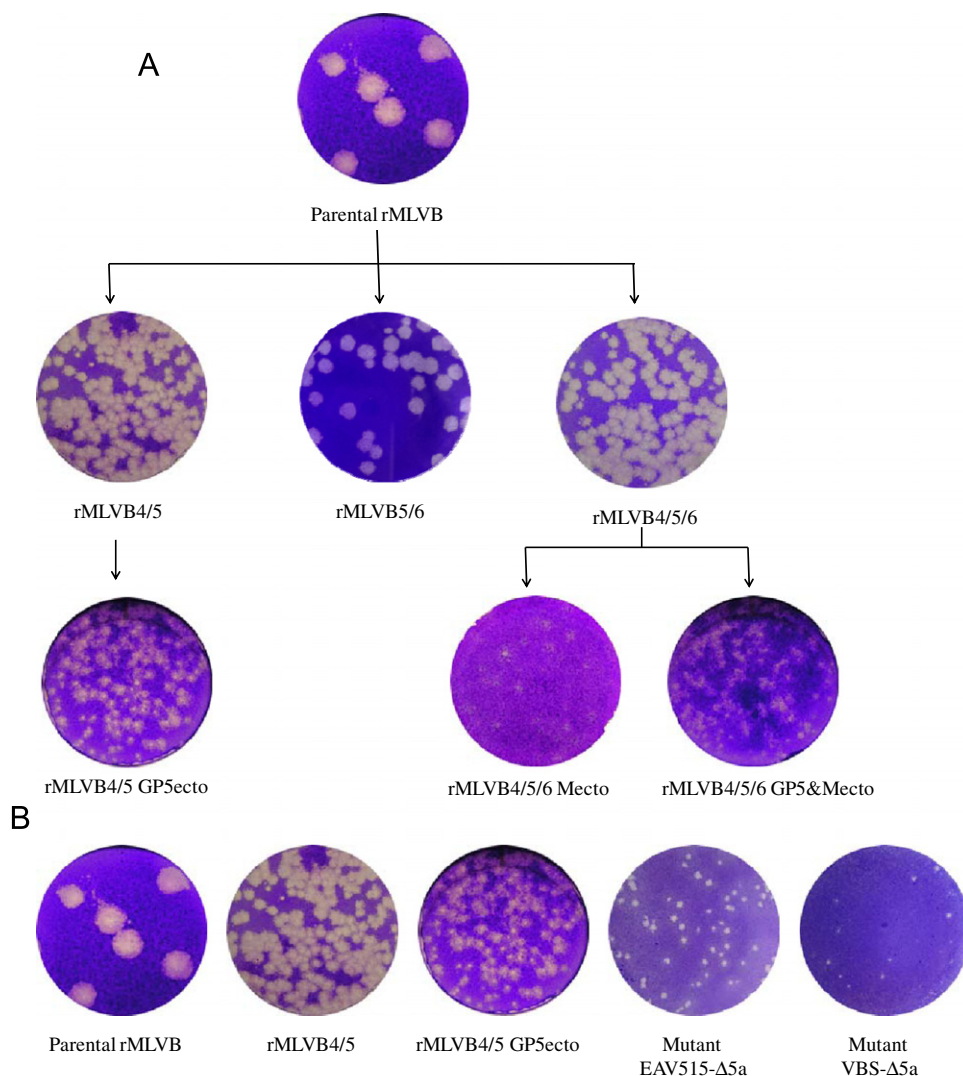


Fig. 4. (A) Comparison of plaque morphology on equine endothelial cells (EEC) of parental rMLVB, recombinant rMLVB4/5, rMLVB5/6 and rMLVB4/5/6 and chimeric rMLVB4/5 GP5ecto, rMLVB4/5/6 Mecto and rMLVB4/5/6 GP5&Mecto viruses. (B) Illustration of plaque morphology of ORF5a knockout mutants (EAV515-Δ5a, VBS-Δ5a) as compared to the parental rMLVB, recombinant rMLVB4/5 and chimeric rMLVB4/5/ GP5ecto viruses. The insertion of 8–10 amino acids into the ORF5a protein of EAV did not cripple the virus to the same extent as the knockout mutants. The mutant EAV515-Δ5a and the mutant VBS-Δ5a plaque morphology figures were reproduced from Firth et al., (2011) with permission.

2002). As we have shown previously, recombinant EAV (A45.C2) containing the GP5 ectodomain of PRRSV (IAF-Klop) is not neutralized by any EAV specific MAbs or by the polyclonal equine antisera (Balasuriya et al., 2004). Therefore, it will be interesting to determine the replication and immunogenic kinetics of these viable EAV/PRRSV chimeras in pigs.

Effect of ORFs4/5 and ORFs5/6 separations on ORF5a protein and GP5 proteins

Recently, a novel small ORF5a protein (encoded by ORF5a) which overlaps with the 5' end of ORF5 has been identified in both EAV and PRRSV (Firth et al., 2011; Johnson et al., 2011). The effect of ORF4/5 separation on ORF5a protein and GP5 was determined by comparative amino acid sequence analysis (Fig. 3). Insertion of the 24 nucleotide sequence to separate ORFs4/5 in the prMLVB vector resulted in replacement of 4 amino acids (NAIY [a.a. 12–15]) with 12 amino acids (SAICELKOHAVH [a.a. 12–23]); this expanded the length of the EAV ORF5a protein by 8 amino acids. The insertion of 24 nucleotides did not affect the transcriptional regulatory sequence (TRS) upstream of ORF5

of rMLVB4/5. It also did not alter the coding sequences of GP5 and M proteins nor the TRS upstream of ORF6. The growth curve and plaque morphology of rMLVB4/5 indicated that separation of ORF4/5/6 had some negative impact on the recombinant viruses. However, these changes did not have a significant crippling effect on the recombinant virus. Previously it was reported that a complete knock out of the ORF5a protein in two infectious cDNA clones pEAV515, a derivative of pEAV030 (van Dinten et al., 1997) and pEAVrVBS (Balasuriya et al., 2007), had a significant crippling effect on the progeny viruses, which produced lower titered virus and pinpoint plaques (Firth et al., 2011). In the current study, insertion of 8 random amino acids to the ORF5a protein of EAV ORF4/5 recombinant virus did not cripple the virus to the same extent compared to the ORF5a knockout mutants (Fig. 4B). Following the replacement of EAV GP5 N-terminal ectodomain (a.a. 1–114) with PRRSV IA1107 GP5 N-terminal ectodomain (a.a. 1–64), the new EAV/PRRSV fusion ORF5a protein consists of 11 amino acids (MFFYDWYVGLN) from EAV ORF5a protein followed by 10 random amino acids (SAICELKQHV) encoded by the nucleotide sequence that was inserted for the separation of ORFs4/5 and the PRRSV ORF5a amino acid sequence (a.a. 22–62) corresponding

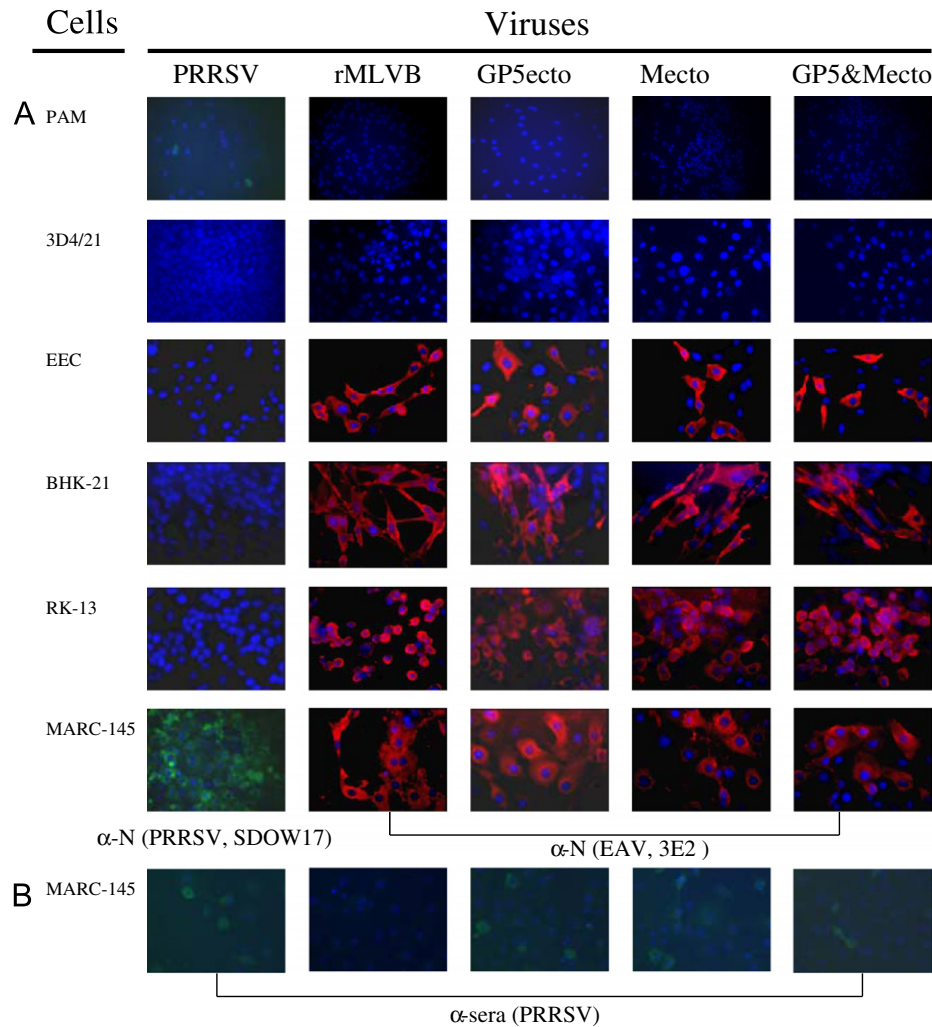


Fig. 5. Determination of cellular tropism of EAV–PRRSV chimeric viruses using indirect immunofluorescence assay (IFA). Various cell lines used in this study are listed on the left side of the figure. The names of the P0 viruses used to infect cells are identified at the top center. The primary antibodies or anti-sera used in IFA are shown at the bottom of the figure. In (A) PRRSV infected cells (first column) were stained with FITC-conjugated SDOW17 MAb which was used to stain PRRSV N protein (green). The remaining columns show the chimeric virus-infected cell lines stained with EAV MAb (3E2) against N protein followed by Texas-red conjugated secondary antibody staining (red). In (B), MARC-145 cells infected with PRRSV or rMLVB or chimeric viruses were stained with pig polyclonal anti-PRRSV sera followed by FITC-conjugated secondary antibody staining. Cell nucleus was stained by Hoechst 33342 (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3

Cellular tropism of various chimeric viruses to different cell types as compared to PRRSV and EAV.

	PRRSV	EAV rMLVB	rMLVB4/5 GP5ecto	rMLVB4/5 GP5full	rMLVB4/5/6 Mecto	rMLVB5/6 Mfull	rMLVB4/5/6 GP5&Mecto
PAM ^a	+	–	–	–	–	–	–
3D4/21	–	–	–	–	–	–	–
Marc 145	+	+	+	–	+	–	+
EEC	–	+	+	–	+	–	+
BHK-21	–	+	+	–	+	–	+
RK-13 HP	–	+	+	–	+	–	+
Notes				Non-infectious		Non-infectious	

^a Porcine alveolar macrophage.

to amino acids 6 to 46 of the PRRSV ORF5a protein. The recombinant EAV/PRRSV ORF5a fusion protein containing the PRRSV GP5 ectodomain was 16 amino acids longer (46 versus 62 a.a.) than the authentic protein and its first 11 amino acids were identical to those in the EAV ORF5a protein. Interestingly, the chimeric viruses with this ORF5a protein (rMLVB4/5 GP5ecto and rMLVB GP5&Mecto) were stable and replicated to titers of

9.5×10^5 pfu/ml. Taken together, these data further confirmed the importance of the ORF5a protein in virus infection as well as the plasticity of this region in having able to accommodate small stretches of additional nucleotides. This observation is further supported by previous attempts to engineer an EAV vectored vaccine by fortuitously introducing a 24 nt insertion to disrupt the ORF4/5 overlap (de Vries et al., 2000). Furthermore, the

difference in length of the ORF5a protein among arteriviruses (EAV 59 codons, PRRSV-NA 46 or 56 codons, PRRSV-EU 43 codons, LDV 47 codons and SHFV 64 codons) and spontaneous insertion of 12 nt into ORF5a during 70 serial passages of PRRSV-NA strain (Han et al., 2009) confirm the plasticity of this region of EAV and other arteriviruses.

In summary, in this study, we have generated three viable EAV/PRRSV chimeric viruses (rMLVB4/5 GP5ecto, rMLVB4/5/6 Mecto and rMLVB4/5/6 GP5&Mecto). The chimeric viruses containing PRRSV M ectodomain are novel. We have used these three viruses to demonstrate unequivocally that the ectodomains of GP5 and M are not the major determinants of cellular tropism, further supporting the recent findings that the minor envelope proteins GP2, GP3, GP4 and E are the critical proteins in mediating cellular tropism (Tian et al., 2012).

Material and methods

Cells and viruses

The cell lines used in the study included equine pulmonary artery endothelial cells (EECs, P12 to P22) (Hedges et al., 2001), high passage rabbit kidney-13 cells (RK-13 KY, P399 to P409), baby hamster kidney cells (BHK-21 [ATCC CRL-12072], Manassas, VA), MARC-145 cells and porcine macrophage cells 3D4/21 (ATCC CRL-2843, Manassas, VA). The EECs were maintained in DMEM with high glucose supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), penicillin, streptomycin, L-glutamine and non-essential amino acids (Life Technologies, Grand Island, NY). RK-13 KY, BHK-21 and MARC-145 were maintained at 37 °C in Eagle's minimum essential medium with 10% ferritin-supplemented bovine calf serum (Hyclone, Logan, UT) penicillin, streptomycin, amphotericin B, and sodium bicarbonate (Life Technologies, Grand Island, NY). In addition, porcine alveolar macrophages (PAM) were isolated and stored at –80 °C until used (kindly provided by Dr. Kay Faaberg, USDA-ARS, Ames, IA). The PAM cells were maintained in Dulbecco's MEM supplemented with 5% fetal bovine serum and 5% antibiotic/antimycotic (Life Technologies, Grand Island, NY). The PRRSV IA1107 strain was kindly provided by Fort Dodge Animal Health Laboratories (now Pfizer Animal Health Inc., Kalamazoo, MI). This strain was isolated from the lung of a pig that died of PRRS. Working virus stock of this strain was made by passaging once in MARC-145 cells.

Construction of EAV/PRRSV chimeric infectious cDNA clones by swapping ORF5 and 6 from PRRSV

The development and characterization of an infectious cDNA clone (prMLVB) of the modified live vaccine strain of EAV (ARVAC[®]) that was used as the backbone to develop the chimeric viruses has been previously described (Zhang et al., submitted for publication) (GenBank accession number FJ798196). The generation of chimeric infectious cDNA clones was performed by a previously described method with some modifications (de Vries et al., 2000). Briefly, three plasmid constructs were generated by separating the overlapping regions between ORFs 4/5 and 5/6 of the prMLVB infectious cDNA clone of EAV (Fig. 1). The unique XbaI site at nt position 14530–14535 was knocked out in the prMLVB clone to generate the shuttle vector prMLVB-Xbalko to facilitate the cloning strategy. The ORFs 4/5 overlapping region (11144CAATGCTATCTATGATT₁₁₁₆₀) was first removed from the prMLVB-Xbalko clone by site-directed mutagenesis and then the 5'CAGTGCTATCTGTGAacttaagcaatgctgtccatgaTT3' sequence was inserted into the corresponding region to obtain the prMLVB4/5 (GenBank accession number JQ844153) in which the ORF4 and ORF5 overlapping regions were separated. A unique restriction

site AflII (5'CTTAAG3') was introduced into the region separating ORF4 and ORF5. Similarly, the ORFs 5/6 overlapping region 11899GTATGGGAGCCATAGAT₁₁₉₁₅ was removed from the prMLVB-Xbalko clone by insertion of the nucleotides 5'GTGTGGGAGCCATAGagcggcgcccaatgggagccatagAT3' into the corresponding region to obtain the clone prMLVB5/6 (GenBank accession number JQ844154). A unique restriction site NotI (5'GCGGCCGC3') was introduced into the region separating ORF5 and ORF6 to facilitate the next cloning step. Subsequently, using a similar approach, a third construct was developed in which both 4/5 and 5/6 ORFs were separated (prMLVB4/5/6, GenBank accession number JQ844155). These three clones were used to generate various EAV/PRRSV chimeric viruses by swapping ORF5 and ORF6 (N-terminal ectodomains and as well as full-length) from PRRSV IA1107 strain.

The GP5 N-terminal ectodomain of PRRSV IA1107 strain (nt 11788–11979; numbered according to GenBank accession number U87392) was reverse transcribed with primer PVGP5ectoN followed by PCR amplification using primers PVGP5ectoP and PVGP5ectoN (Supplementary Table 1) using a commercial one-step RT-PCR kit (Qiagen, Valencia, CA). The PRRSV GP5 ectodomain was then cloned into the plasmid prMLVB4/5 using unique restriction sites AflII and EcoRI to obtain the recombinant plasmid prMLVB4/5 GP5ecto (GenBank accession number JQ844156). The GP5 full-length domain of PRRSV IA1107 (nt 13788–14390) was RT-PCR amplified with primer PV15364N followed by PCR amplification using primers PVGP5ectoP and PVGP5fullN and cloned into prMLVB4/5 with restriction sites NotI and XbaI to obtain the recombinant plasmid prMLVB4/5 GP5full. Similarly, the M full-length domain of PRRSV IA1107 (nt 14375–14899) was reverse transcribed with primer PV15364N and PCR amplified using primers PVMfullP and PVMfullN and cloned into prMLVB5/6 with restriction sites NotI and XbaI to generate the chimera construct prMLVB5/6 Mfull. Because of the short length of the M protein N-terminal ectodomain sequence, the N-terminal ectodomain of PRRSV IA1107 M protein (nt 14375–14425) was directly inserted into the shuttle vector prMLVB-Xbalko4/5/6RemMecto, in which the M protein N-terminal ectodomain of EAV was removed via site-directed mutagenesis, using primer EAVinsPVMectoP and EAVinsIA1107MectoN to generate prMLVB4/5/6 Mecto (GenBank accession number JQ844157). The specific nucleotide locations were identified according to the North American VR2332 strain of PRRSV sequence (GenBank accession number U87392). The prMLVB4/5/6 Mecto was further used as a vector to introduce the PRRSV GP5 N-terminal ectodomain sequences using the above described cloning strategies to generate prMLVB4/5/6 GP5&Mecto plasmid (GenBank accession number JQ844158). The nucleotide sequences of the primers used in this study are listed in Supplementary Table 1. The panel of chimeric constructs between the EAV and PRRSV major envelope protein coding genes is shown in Fig. 1.

Sequencing of the plasmid constructs

The region spanning from ORFs 4–7 of prMLVB4/5, prMLVB5/6, and prMLVB4/5/6 plasmids, as well as all five chimeric plasmids were PCR amplified and sequenced to confirm their authenticity. Briefly, each of the plasmids was amplified using primers that were specific for the 5' end of the ORFs 4 and the 3' end of ORF7 (10763P and 12568N primers [Supplementary Table 1]; numbered according to the published sequence of the VB strain of EAV, GenBank accession number DQ846750) using the high-fidelity proofreading Pfu Turbo DNA polymerase (Agilent Technology, Santa Clara, CA) according to a previously described protocol (Zhang et al., 2008). Both sense and antisense strands of the chimeric plasmids were sequenced using EAV specific primers 10763P, 11691N, 11557P and 12568N (Supplementary

Table 1) at MWG Operon, Huntsville, AL. The sequence data were analyzed using Aligner version 1.5.2 (CodonCode, Dedham, MA) software program. Comparative nucleotide and amino acid sequence analysis of the 3'-end of all the plasmid were performed with Vector NTI (Life Technologies, Carlsbad, CA) software.

In vitro transcription (IVT) and generation of recombinant chimeric viruses

Run-off viral RNA transcripts from each recombinant/chimeric infectious clone after linearization with restriction enzyme XhoI were generated as previously described (van Dinten et al., 1997). Eighty micrograms of full-length IVT RNA generated from each recombinant and chimeric plasmid construct were transfected into BHK-21 cells by electroporation as previously described (Balasuriya et al., 1999; van Dinten et al., 1997). After electroporation, cells were incubated at room temperature for 10 min and mixed with 12 ml of complete BHK-21 medium warmed to room temperature. The cells were seeded into 10-cm-diameter tissue culture plates (Falcon) and incubated at 37 °C for 48–72 h until cytopathic effect (CPE) was evident. The tissue culture fluid (TCF) supernatant collected after IVT RNA transfection was designated P0 virus and used for further characterization. The stability of each recombinant/chimeric virus was determined by 10 sequential passages in BHK-21 cells and sequencing the RNA extracted from passages 0, 5 and 10 (see below).

RT-PCR amplification and sequencing of ORFs 5–6 of recombinant viruses

The authenticity of each recombinant/chimeric virus stock was determined by RT-PCR amplification and sequencing of the ORFs 5–6 region from RNA that was isolated from TCF from P0, P5 and P10 of each virus as previously described (Balasuriya et al., 1999). Briefly, RNA was directly isolated from the TCF using QIAmp viral RNA isolation kit (Qiagen, Valencia, CA). The purified RNA was treated with DNase I (Life Technologies, Grand Island, NY) for 30 min at 37 °C to remove any contaminating plasmid DNA before RT-PCR amplification. The ORFs 5–6 region was RT-PCR amplified using EAV specific primers 10763P and 12568N (Supplementary Table 1). The PCR was also performed with a non-RT step to eliminate the possibility of amplifying any remaining plasmid DNA. Sequence data were analyzed as described previously.

Antibodies

The monoclonal antibodies (MAbs) 3E2 and SDOW-17 (Rural Technology Inc., Brookings, SD) against N proteins of EAV and PRRSV, respectively, have been previously described (MacLachlan et al., 1998; Nelson et al., 1993). Swine polyclonal anti-PRRSV serum (PHGB 2008 ID#1467) was obtained from a PRRSV infected pig with high PRRSV neutralizing activity. Commercial fluorescent labeled anti-swine (FITC conjugated anti-swine IgG [Santa Cruz Biotechnology, Santa Cruz, CA]) and anti-mouse (Texas red-conjugated goat anti-mouse IgG [Thermo Scientific, Rockford, IL]) were used in indirect immunofluorescence assays.

Indirect immunofluorescence assay (IFA)

Various cell types (EEC, BHK-21, RK-13, PAM, 3D4/21, MARC-145) grown in Lab-Tek™ eight-well chamber slides (Nalge-Nunc, Rochester, NY) were either mock infected or infected with various recombinant chimeric viruses (P0) and incubated at 37 °C for 36–48 h. The slides were fixed with 3% paraformaldehyde in PBS (pH=7.4) and stained with EAV N protein specific MAb 3E2 followed by secondary Texas red-conjugated goat anti-mouse

IgG staining. The BHK-21, RK13, EECs, MARC-145, PAM and 3D4/21 cells infected with PRRSV were stained with FITC conjugated MAb against PRRSV N protein (SDOW-17) (MacLachlan et al., 1998; Nelson et al., 1993). Since PRRSV GP5 and M protein specific antibodies were not available, swine polyclonal anti-PRRSV serum was used to detect the chimeric PRRSV proteins in infected cells. EAV antibodies were incubated for 1 h at room temperature prior to secondary antibody staining. PRRSV MAb against N protein (SDOW-17) or swine polyclonal anti-PRRSV serum was incubated for 1 h at 37 °C followed by overnight incubation at 4 °C to enhance the antibody signal.

In vitro growth characteristics of EAV-PRRSV chimeric viruses

BHK-21 cells grown in six-well plates were inoculated with EAV-PRRSV chimeric viruses (P0) as well as parental rMLVB virus and its derivatives (rMLVB4/5, rMLVB5/6 and rMLVB4/5/6) at an MOI of 1 and incubated at 37 °C for 1 h. The inocula were removed and cells were washed with PBS (pH=7.4) three times to remove unbound virus before being overlaid with 4 ml of EMEM. At 0, 6, 12, 24, 48, 72 h post-infection, TCFs were collected and virus titers were determined by plaque assays in RK-13 cells as previously described (Wilson et al., 1962).

Plaque morphology

The plaque morphology of the recombinant viruses was determined by plaque assays in EECs. Briefly, confluent monolayers of EECs in 6-well culture plates were infected with serial 10-fold dilutions of each recombinant/chimeric virus (rMLVB, rMLVB4/5 and rMLVB4/5/6, rMLVB4/5 GP5ecto, rMLVB4/5/6 Mecto and rMLVB4/5/6 GP5&Mecto) virus in duplicate. Following 1 h incubation, 0.75% CMC (cboxymethylcellulose)–DMEM was added to each well and cells were incubated at 37 °C for 96 h (McCollum et al., 1961). Plaques were stained with 1% formalin–crystal violet solution containing 1% formaldehyde at 96 h post-infection and titers were expressed in pfu/ml.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.virol.2012.05.022>.

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