Development and characterization of an infectious cDNA clone of the virulent Bucyrus strain of Equine arteritis virus


1Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, CA 95616, USA
2Molecular Virology Laboratory, Department of Medical Microbiology, Leiden University Medical Center, Leiden, The Netherlands
3Department of Biology, University of Texas at San Antonio, TX 78249, USA
4Department of Veterinary Science, Gluck Equine Research Center, University of Kentucky, Lexington, KY 40546, USA

Strains of Equine arteritis virus (EAV) differ in the severity of the disease that they induce in horses. Infectious cDNA clones are potentially useful for identification of genetic determinants of EAV virulence; to date, two clones have been derived from a cell culture-adapted variant of the original (Bucyrus) isolate of EAV, and it has previously been shown that recombinant virus derived from one of these (rEAV030) is attenuated in horses. A complete cDNA copy of the genome of the virulent Bucyrus strain of EAV has now been assembled into a plasmid vector. In contrast to rEAV030, recombinant progeny virus derived from this clone caused severe disease in horses, characterized by pyrexia, oedema, leukopenia, high-titre viraemia and substantial nasal shedding of virus. The availability of infectious cDNA clones that produce recombinant viruses of different virulence to horses will facilitate characterization of the virulence determinants of EAV through reverse genetics.

Equine arteritis virus (EAV) is the prototype virus in the family Arteriviridae (order Nidovirales) (Cavanagh, 1997; Snijder & Meulenberg, 1998). The EAV virion is an enveloped, spherical, 50–65 nm diameter particle with an icosahedral core that contains a single-stranded, positive-sense RNA molecule of approximately 12.7 kb (Snijder & Meulenberg, 1998; Snijder & Meulenberg, 2001). The EAV genome includes 5′ and 3′ untranslated regions (UTRs) and nine open reading frames (ORFs; den Boon et al., 1991; Snijder & Meulenberg, 1998). The two most 5′-proximal ORFs (1a and 1b) occupy approximately three-quarters of the genome and encode two replicate polyproteins (pp1a and pp1ab). Following genome translation, the two replicate precursor proteins are processed extensively to yield at least 12 non-structural proteins (nsp 1–12; Snijder & Meulenberg, 1998; van Dinten et al., 1999; Ziebuhr et al., 2000). ORFs 2–7 are located in the 3′-proximal quarter of the genome and encode seven structural proteins of the virus: the envelope proteins E, GP2b (formerly known as G5), GP3, GP4, GP5 (formerly G4L) and M, and the nucleocapsid protein (N; de Vries et al., 1992; Snijder & Meulenberg, 2001; Snijder et al., 1999). Three of the minor envelope proteins (GP2b, GP3 and GP4) form a heterotrimer in the EAV particle, and the M and GP5 proteins form a disulfide-linked heterodimer (de Vries et al., 1995; Wierenga et al., 2004). The structural proteins are produced from a 3′-coterminally nested set of six subgenomic miRNAs, which also contain a common 5′ leader sequence derived from the 5′ end of the genome [reviewed by Pasternak et al. (2006)].

There is only one known serotype of EAV and all strains evaluated thus far are neutralized by polyclonal antiserum raised against the virulent Bucyrus strain (Balasuriya & MacLachlan, 2004; Balasuriya et al., 1995, 1997, 2004; Chirnside et al., 1995; Deregt et al., 1994; Glaser et al., 1995). However, field strains of EAV can often be distinguished on the basis of their neutralization phenotype with polyclonal...
antiseras and mAbs. Likewise, geographically and temporally distinct strains of EAV differ in the severity of the clinical disease that they induce and in their abortigenic potential (Balasuriya et al., 1998, 1999a; McCollum & Timoney, 1998; McCollum et al., 1998; Murphy et al., 1992; Patton et al., 1999; Timoney & McCollum, 1993). The pathogenesis of equine viral arteritis (EVA) has been studied both by the experimental inoculation of horses with strains of EAV of different virulence and by evaluation of natural outbreaks of EVA (Balasuriya et al., 2002; Cole et al., 1986; Fukunaga et al., 1981; Jones, 1969; MacLachlan et al., 1996; McCollum, 1981, 1986; McCollum & Timoney, 1998; McCollum et al., 1971, 1998). Most strains of EAV cause only subclinical or asymptomatic infection of horses, whereas some cause disease of varying severity. The genetic basis of this variation in virulence phenotype amongst EAV strains has not been characterized.

To date, two infectious cDNA clones of the original Bucyrus strain of EAV have been described, which were both derived from a highly cell culture-adapted variant (de Vries et al., 2000; Glaser et al., 1998; van Dinten et al., 1997). We have shown previously that the recombinant virus (rEAV030) derived from one of these clones (pEAV030 (GenBank accession no. Y07862); van Dinten et al., 1997) is attenuated in horses (Balasuriya et al., 1999b). In this report, we describe the construction of a stable full-length cDNA clone of the horse-adapted, highly virulent Bucyrus strain (VBS) of EAV. RNA transcribed from this plasmid was infectious upon transfection into mammalian cells, as shown by expression of viral proteins and production of infectious progeny virus. The recombinant virus derived from this infectious cDNA clone (rVBS) caused severe disease in experimentally inoculated horses.

The VBS strain of EAV (ATCC VR-796) was propagated in rabbit kidney 13 (RK-13; ATCC CCL37) cells without plaque purification and a stock was made and stored at −80 °C. The virulence to horses of this virus stock was confirmed by experimental infection of a horse (MacLachlan et al., 1996). Genomic RNA was isolated from tissue-culture fluid by using QIAamp viral RNA purification columns (QIAamp viral RNA kit; Qiagen). The first-strand cDNA was synthesized by using SuperScript II RNase H− reverse transcriptase (Invitrogen) and gene-specific primers. Long PCR was carried out according to the manufacturer’s instructions with the Expand Long Template PCR system (Boehringer Mannheim). This system utilizes a unique enzyme mixture containing Taq DNA polymerase (5′–3′ polymerase activity) and Pwo DNA polymerase (3′–5′ proofreading ability). Synthetic oligonucleotide primers were designed according to the published sequence of EAV (GenBank accession no. X53459; den Boon et al., 1991) and used for PCR amplification and sequencing. A full-length cDNA clone of the VBS virus was assembled from two overlapping PCR fragments flanked by unique restriction sites [12704 bp viral cDNA flanked by Xbal (5′) and Xhol (3′) sites was generated by cloning into the pTRSB vector] (McKnight et al., 1996; see Supplementary Fig. S1, available in JGV Online). The resulting plasmid, pEAVrVBS (GenBank accession no. DQ846751), contained the complete EAV cDNA downstream of the bacteriophage T7 RNA polymerase promoter. At the 3′ end of the viral insert, a 20 nt poly(A) tail preceded the unique Xhol restriction site that was used for linearization prior to run-off in vitro transcription. Although a high-fidelity, thermostable, proof-reading DNA polymerase was used to minimize the inadvertent introduction of mutations during PCR amplification of the viral cDNA, sequence analysis of the full-length clone revealed six amino acid changes compared with the consensus sequence of the parental VBS virus (GenBank accession no. DQ846750; J. Zhang, W. H. McCollum, U. B. R. Balasuriya & P. J. Timoney, unpublished results; Table 1). Five of these amino acid changes were located in the non-structural proteins (ns2, 9 and 10) of the recombinant virus, whereas the other amino acid substitution was located in the GP5 structural protein. The rVBS virus differed from the rEAV030 virus in 22 aa and 12 of these differences were located in the structural proteins. The rVBS virus also had 25 and 31 non-coding nucleotide changes compared with the parental VBS and rEAV030 viruses, respectively (see Supplementary Table S1, available in JGV Online).

Capped RNA was in vitro-transcribed from the Xhol-linearized full-length cDNA clone (Balasuriya et al., 1999b) and transfected into either baby hamster kidney cells (BHK-21; ATCC CCL10) or equine pulmonary artery endothelial cells (ECs; Hedges et al., 2001; Moore et al., 2002) by electroporation as described previously (Balasuriya et al., 1999b). The cells were seeded onto 10 cm diameter plates (Falcon) and incubated at 37 °C for 3 days until complete cytopathic effect (CPE) was evident. Cell-culture supernatant was harvested and centrifuged at 1600 g for 10 min at 4 °C. The supernatant was aliquotted and stored at −80 °C. Titres of the recombinant virus (rVBS) were determined by both end-point dilution (Reed & Muench, 1938) and plaque assays [2.0 × 10^6.25 tissue culture infectious doses (TCID₅₀) ml⁻¹ and 3.25 × 10⁸ p.f.u. ml⁻¹].

An immunofluorescence assay (IFA) was used to detect viral protein synthesis in BHK-21 cells transfected with synthetic full-length RNA. For IFAs, electroporated BHK-21 cells were plated directly onto chamber slides and incubated at 37 °C for 20–22 h. Mono-specific rabbit anti-peptide serum to the nsp2 and nsp7/8 proteins (Snijder et al., 1994) and mAbs to the GP5 and N proteins (Balasuriya et al., 1993, 1997; MacLachlan et al., 1998) were used to detect the production of EAV replicase and structural proteins in transfected cells, as described previously (van der Meer et al., 1998; van Dinten et al., 1997). Fluorescence was detected in 30% of the transfected cells at 20–22 h after transfection (data not shown), and fluorescence intensity and numbers of IFA-positive cells had increased by 48 h. As a negative control, cells were transfected with a mutant RNA containing lethal mutations at nt 4581 (resulting in a 1453 S→R amino acid change in the ORF1a protein) and nt 7332 (2370 E→G in ORF1b). These mutations mapped to conserved
rEAV030) of two of the cleavage sites for the EAV main proteinase in the replicase polyprotein, the nsp6/7 and nsp9/10 junctions, respectively (Ziebuhr et al., 2000). Processing of these sites was previously found to be essential for EAV viability (van Dinten et al., 1999). No positive IFA staining was detected at any time in cells transfected with the mutant RNA (data not shown). These data confirm that the positive IFA signals were derived from replication of the synthetic RNA in transfected cells, resulting in the generation of progeny virus that subsequently spread to neighbouring cells and initiated additional cycles of infection.

### Table 1. Amino acid differences between rVBS and the parental VBS and rEAV030

<table>
<thead>
<tr>
<th>ORF</th>
<th>Protein length (aa)</th>
<th>Amino acid differences between:</th>
<th>Parental VBS and rVBS*</th>
<th>rEAV030 and rVBS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORF1ab</td>
<td>Non-structural proteins:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>225–9751</td>
<td>3175</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nsp1:</td>
<td>Met1–Gly260 (260)</td>
<td></td>
<td>–</td>
<td>51 M→V</td>
</tr>
<tr>
<td>nsp2:</td>
<td>Gly261–Gly831 (571)</td>
<td></td>
<td>–</td>
<td>382 G→D</td>
</tr>
<tr>
<td>nsp3:</td>
<td>Gly832–Glu1064 (233)</td>
<td></td>
<td>–</td>
<td>559 N→S</td>
</tr>
<tr>
<td>nsp4:</td>
<td>Gly1065–Glu1268 (204)</td>
<td></td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>nsp5:</td>
<td>Ser1269–Glu1430(162)</td>
<td></td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>nsp6:</td>
<td>Gly1431–Glu1452 (22)</td>
<td></td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>nsp7:</td>
<td>Ser1453–Glu1677 (225)</td>
<td></td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>nsp8:</td>
<td>Gly1678–Asn1727 (50)</td>
<td></td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>nsp9:</td>
<td>Gly1678–Glu2370 (693)</td>
<td></td>
<td>1970 D→G</td>
<td></td>
</tr>
<tr>
<td>nsp10:</td>
<td>Ser2371–Gln2837 (467)</td>
<td></td>
<td>2000 V→A</td>
<td></td>
</tr>
<tr>
<td>nsp11:</td>
<td>Ser2838–Glu3056 (219)</td>
<td></td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>nsp12:</td>
<td>Gly3057–Val3175 (119)</td>
<td></td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Structural proteins:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORF2a</td>
<td>(9751–9954)</td>
<td></td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>ORF2b</td>
<td>(9824–10507)</td>
<td></td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>ORF3</td>
<td>(10306–10797)</td>
<td></td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>ORF4</td>
<td>(10700–11158)</td>
<td></td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>ORF5</td>
<td>(11146–11913)</td>
<td></td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>ORF6</td>
<td>(11901–12389)</td>
<td></td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>ORF7</td>
<td>(12313–12645)</td>
<td></td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Total no. amino acid changes</td>
<td></td>
<td></td>
<td>6</td>
<td>22</td>
</tr>
</tbody>
</table>

The replication kinetics of three EAV strains (rVBS, wild-type VBS and rEAV030) were compared in confluent monolayers of ECs by using one-step growth curves and plaque morphology/size (Moore et al., 2002, 2003). Briefly, six-well plates containing ECs were inoculated in triplicate with each virus at an m.o.i. of 5. Virus was adsorbed for 1 h and cultures were then washed three times with Eagle’s minimal essential medium (EMEM) and supplemented with complete EC medium. Cultures were incubated at 37 °C in 5% CO₂, and tissue-culture fluid (TCF) samples were harvested at 1, 6, 12, 24, 36, 48 and 54 h after infection. Titres of EAV in TCF samples were determined by plaque...
using sterile gauze sponges at the end of a stainless-steel wire, as described previously (Balasuriya et al., 1999b). Virus isolation from mononuclear cells, plasma and nasal swabs was attempted as described previously (Balasuriya et al., 1999b). All four horses developed severe clinical signs of viral arteritis, including high fever (38.9–40.6 °C) for 5–6 days, severe lymphopenia, petechial and ecchymotic haemorrhages in the oral mucous membranes and skin eruptions (hives) on the neck, shoulder and along the back (Figs 1 and 2). The horses were depressed and developed a serous nasal discharge and supraorbital and leg oedema of variable severity. Virus was isolated from the nasal swabs, plasma and PBMCs collected from all four horses, and all developed high-titre viraemia ($6 \times 10^{5.9} – 1 \times 10^{5.9}$ p.f.u. ml$^{-1}$ in PBMCs and $1 \times 10^{5.0} – 1 \times 10^{5.9}$ p.f.u. ml$^{-1}$ in plasma at 4 and 6 days p.i.) and nasal shedding of EAV ($4 \times 10^{4}$ p.f.u. ml$^{-1}$ at 4 and 6 days p.i.). EAV was isolated for 28–56 days p.i. from buffy coat and for up to 12 days p.i. from plasma, and nasal shedding was detected until 10–12 days p.i. All four horses seroconverted to EAV by 6–8 days p.i. and neutralizing-antibody titres in their serum increased to $> 512$ by 14 days p.i. (data not shown). Virus clearance coincided with the appearance of neutralizing antibodies. The master sequence of rVBS was stable during in vivo replication, as direct RT-PCR sequencing of ORFs 2–7 from RNA extracted from PBMCs collected at 6

### Assay on RK-13 Cells

For plaque-morphology determination, each virus was assayed for 1 h onto confluent monolayers of ECs in 25 cm$^2$ tissue-culture flasks and the cultures were then overlaid with complete growth medium containing 0.75% carboxymethylcellulose (Sigma). The monolayers were incubated for 96 h and fixed and stained with 1% crystal violet. There was no significant difference in the growth kinetics of rVBS compared with those of the parental wild-type VBS or the rEAV030 virus, which represents a highly cell culture-adapted strain of the VBS virus. rVBS and its VBS parent produced identical plaques in ECs (mean diameter, 3.2 mm), confirming that replication and spread of these two viruses were indistinguishable in ECs. Interestingly, although the growth kinetics of the avirulent rEAV030 virus were similar to those of VBS and rVBS, rEAV030 produced significantly larger (4.9 mm) plaques in ECs, which were similar in size to those of the modified live virus (MLV) vaccine strain (ARVAC; Moore et al., 2002, 2003), which is also an extensively cell culture-passaged derivative of the VBS virus. It has previously been demonstrated that virulent strains of EAV generally produce significantly larger plaques in ECs than avirulent strains (Moore et al., 2003). In summary, the plaques induced by rVBS infection in ECs are indistinguishable from those of the virulent parental VBS virus and differ from the very large plaques produced by both the MLV vaccine and the avirulent rEAV030 virus. The fact that highly cell culture-adapted strains produce very large plaques in ECs suggests that these two viruses are adapted to growth in cell culture and that other nucleotide and amino acid changes may be responsible for their altered phenotypic change. Furthermore, the mechanism of attenuation of the MLV vaccine and rEAV030 is probably different from that of the other avirulent field stains of EAV that all consistently produce small plaques in ECs (Moore et al., 2003).

### Experimental Infection

The virulence phenotype of rVBS was determined by experimental infection of horses. Briefly, two mares and two geldings, seronegative for EAV by serum neutralization and Western immunoblotting assays, were housed in an isolation facility. Each horse was inoculated intranasally with $3.25 \times 10^6$ p.f.u. rVBS ml$^{-1}$ that was delivered in 4.0 ml EMEM by using a fenestrated catheter. The horses were monitored twice daily for 4 weeks for clinical manifestations of EVA. Whole blood for haematology [in buffered sodium citrate (Monoject; Becton Dickinson) and Vacutainer EDTA (Becton Dickinson)] was collected at 0, 2, 4, 6, 8, 10, 12 and 14 days post-infection (p.i.) for complete and differential blood counts. Plasma for virus isolation (Vacutainer citrate) was collected at 0, 2, 4, 6, 8, 10, 12, 14, 21, 28, 35 and 42 days p.i. Whole blood samples were also collected into Vacutainer CPT cell preparation tubes (Becton Dickinson) for the separation of peripheral blood mononuclear cells (PBMCs) for virus isolation and viral RNA extraction. Nasopharyngeal swabs were obtained from all horses at 0, 2, 4, 6, 8, 10, 12, 14, 21 and 28 days p.i. by

---

http://vir.sgmjournals.org

---

**Fig. 1.** Body temperature (a) and peripheral blood lymphocyte counts (b) of four horses (TDE, ACH, KAS and PGI) after inoculation with recombinant virus (rVBS) derived from the infectious cDNA clone.
and 10 days p.i. of two horses confirmed all of the changes listed in Table 1.

The parental virus (VBS) on which this infectious cDNA clone was based is considered the prototype strain of EAV and causes severe clinical disease in inoculated horses (MacLachlan et al., 1996; McCollum & Timoney, 1998). Most laboratory strains of EAV used around the world, including the parental virus of rEAV030, are highly cell culture-passaged derivatives of VBS. Specifically, the parental virus of the pEAV030 infectious cDNA clone was passaged extensively in cell culture prior to end-point dilution and plaque purification in African green monkey kidney (Vero) cells and propagation of virus stocks (van Dinten et al., 1997). We have shown previously that rEAV030 is highly attenuated (Balasuriya et al., 1999b), as horses inoculated with it developed only transient fever and moderate lymphopenia without other clinical signs of viral arteritis. The horses also had low-titre viraemia (\( \leq 1 \times 10^4 \) p.f.u. ml\(^{-1}\) in PBMCs) and virus was shed only transiently in nasal secretions (\( \leq 1 \times 10^5 \) p.f.u. ml\(^{-1}\)). Furthermore, virus and viral RNA were detected in blood for only 10 and 14 days p.i., respectively. Results of the present study clearly confirm that the rVBS virus is markedly more virulent for horses than rEAV030. Thus, the infectious cDNA clone of the VBS strain of EAV provides a critical reverse-genetics system with which it will be possible to characterize the mechanisms of EAV pathogenesis and virulence.

Acknowledgements

The authors gratefully acknowledge Drs Pamela J. Hullinger, W. David Wilson and Steve Lampman, School of Veterinary Medicine, for excellent animal care. These studies were supported by USDA National Research Initiative Competitive grant 02-35204-11545 and the Center for Equine Health, University of California, Davis, CA, USA, with funds provided by the Oak Tree Racing Association, the State of California Satellite Wagering Fund and contributions by private donors.

References


