A stable full-length cDNA clone of the modified live virus (MLV) vaccine strain of equine arteritis virus (EAV) was developed. RNA transcripts generated from this plasmid (pEAVrMLV) were infectious upon transfection into mammalian cells, and the resultant recombinant virus (rMLV) had 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 by using an allelic discrimination real-time reverse transcription (RT)-PCR assay. In vitro studies revealed that the cloned vaccine virus rMLVB and the parental MLV vaccine virus had identical growth kinetics and plaque morphologies in equine endothelial cells. In vivo studies confirmed that the cloned vaccine virus was very safe and induced high titers of neutralizing antibodies against EAV in experimentally immunized horses. When challenged with the heterologous EAV KY84 strain, the rMLVB vaccine virus protected immunized horses in regard to reducing the magnitude and duration of viremia and virus shedding but did not suppress the development of signs of EVA, although these were reduced in clinical severity. 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.

Equine arteritis virus (EAV) is a single-stranded, positive-sense RNA virus in the family Arteriviridae (genus Arterivirus, order Nidovirales), which also includes porcine reproductive and respiratory syndrome virus (PRRSV), simian hemorrhagic fever virus (SHFV), and lactate dehydrogenase-elevating virus (LDV) of mice (10, 41). The EAV genome is approximately 12.7 kb and includes 5’ and 3’ untranslated regions and 10 known open reading frames (ORFs) (13, 41, 42). The first two ORFs (1a and 1b) are approximately 9.5 kb and encode two replicate polyproteins (pp1a and pp1ab) that are posttranslationally processed to yield at least 13 nonstructural proteins (Nsp1 to Nsp12, including Nsp7α and Nsp7β) required for virus replication and transcription (41, 51, 58). The remaining seven ORFs (2a, 2b, 3, 4, 5, 6, and 7) are approximately 2.9 kb and encode the envelope proteins E, GP2, GP3, GP4, GP5, M, and the nucleocapsid protein (N), respectively (41, 42). Recently, a novel small arterivirus gene (ORF5a) that overlaps the GP5 coding sequence (ORF5) that is important for virus production has been identified in all arteriviruses (13, 19).

EAV is the causative agent of equine viral arteritis (EVA) and is distributed in many equine populations throughout the world (18, 25, 36). While most EAV infections are asymptomatic or subclinical, some infected horses exhibit clinical manifestations characteristic of EVA (15, 47). The consequences of EAV infection include influenza-like illness in adult horses, abortion in pregnant mares, pneumonia and/or enteritis in young foals, and persistent infection in stallions (29, 47). Economic losses attributable to EAV infection include mainly abortion, illness, and death in young foals, the carrier state in stallions, and restricted export markets for carrier stallions as well as virus-infective semen or embryos. Experience over the past 20 years would indicate that EAV infection is of increasing significance to the $102 billion/annum horse industry in the United States (38).
of the current vaccine. An infectious cDNA clone based on the MLV vaccine strain would also be a useful tool to study EAV pathogenesis and to define genetic determinants of EAV virulence/attenuation. Here, we describe the development of a stable full-length cDNA clone of the current MLV vaccine strain of EAV. The in vitro and in vivo features of the recombinant virus derived from this infectious cDNA clone were further characterized.

MATERIALS AND METHODS

Cells and viruses. The baby hamster kidney (BHK-21; ATCC CCL-10; Manassas, VA) and high-passage-number rabbit kidney cell line (RK-13 KY; passage levels 399 to 409) were maintained in Eagle’s minimum essential medium (EMEM; Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), 100 U/ml penicillin-streptomycin (Mediatech, Manassas, VA), and 1 μg/ml amphotericin B (Sigma-Aldrich, St. Louis, MO). Equine pulmonary artery endothelial cells (EECs) (17) were maintained in Dulbecco’s modified essential medium (Mediatech, Manassas, VA) with sodium pyruvate, 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 100 U/ml penicillin-streptomycin (Mediatech, Manassas, VA), and 2 mM L-glutamine (Mediatech, Manassas, VA) (3, 8, 23). The current MLV vaccine strain of EAV (ARVAC, lot number 170123A; Fort Dodge Animal Health, Ford Dodge, IA [now Pfizer Animal Health Inc., Kalamazoo, MI]) was used as the source of virus for construction of the infectious cDNA clone. Sixty archived tissue culture fluid (TCF) (20, 54, 56) samples containing various field and laboratory strains of EAV were used for extraction of RNA for developing the allelic discrimination real-time reverse transcription-PCR (rRT-PCR). The virulent KY84 strain of EAV (EAV KY84) was used as the challenge virus (11, 27, 28, 31).

Construction of infectious cDNA clones of the current MLV vaccine (ARVAC) strain of EAV. A full-length cDNA clone of the MLV vaccine virus was generated by assembling four overlapping PCR fragments that encompass the entire genome of the vaccine virus flanked by XbaI (5′) and XhoI (3′) into the pTRSB vector containing the full-length genome of the cloned vaccine rMLVB (G12423) from the parental MLV vaccine strain as well as field and laboratory strains of EAV (G12423). The primers were designed to ORF7, flanking the silent nucleotide substitution, and the TaqMan MGB probes were tagged with a 6-carboxyfluorescein (FAM) (probe G) or VIC (probe C) label. Primer and probes were as follows: forward primer EVAF7F, 5′ GGGCACAGCCTCA CAAAGTCAACA′; reverse primer EVAR7R, 5′ TGGCTAAGGTCCTCAGGG TC3′; probe G EVAGF7F25F, 5′FAM-ATGGCAGTGGCGAA-MGB-NF Q-3′; and probe C EVAGC7F25F, 5′VIC-TGGCAGGGTCGCGAAAA-MGB-N FQ-3′. The primers and fluorogenic TaqMan MGB probes were designed to amplify and detect a highly conserved region of ORF7 of EAV. This region was identified by comparing 208 EAV ORF7 nucleotide sequences available in GenBank. The tissue culture fluids containing the parental vaccine strain (ARVAC), the cloned vaccine virus (rMLVB), and 60 field and laboratory strains of EAV were used for RNA extraction with the QIAamp viral RNA kit (Qiagen, Valencia, CA). Reactions were performed using the TaqMan one-step RT-PCR master mix (Applied Biosystems [ABI], Foster City, CA) in a 7500 Fast real-time PCR system (ABI). Briefly, 25 μl of RT-PCR mixture for each reaction contained 12.5 μl of 2X master mix without uracil-glycosylase (UNG), 0.625 μl of 40× Multi-Scribe and RNase inhibitor mix, 1.25 μl of 20× primer-probe mix (900 nM forward and reverse primers and 250 nM probes), 5.625 μl of nuclelease-free water, and 5 μl of test sample RNA. The following thermocycling conditions were used under standard mode per the manufacturer’s recommendation: 30 min at 48°C, 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

In vitro growth characteristics and plaque morphology of recombinant viruses. Monolayers of BHK-21 cells and EECs grown in 6-well plates were inoculated with each of the MLV and rMLVB viruses at a multiplicity of infection (MOI) of 5 and incubated at 37°C for 1 h. The inocula were aspirated off and the cell sheets washed three times with PBS (pH 7.4) to remove unbound virus and then overlaid with 4 ml of complete culture medium. This was designated time zero with respect to infection. At 0, 6, 12, 24, 48, and 72 h postinfection, supernatants were harvested and virus titers determined by plaque assay in RK-13 cells as previously described (26). The plaque morphologies/sizes of the recombinant rMLVB virus and the parental MLV vaccine strain were compared in confluent monolayers of EECs as previously described (34, 35).

Experimental vaccination of horses. Eight EAV seronegative horses (mares, age 5 to 6 years old) were randomly assigned to two groups. Group one (n = 2 horses; animal ID numbers C7 and C11) and group two (n = 6 horses; animal ID numbers C12, C17, C19, C24, D2, and D8) horses were inoculated intramuscularly (cervical muscle) with PBS (1 ml) and cloned vaccine virus rMLVB (1.0 × 107 PFU of virus in 1 ml of PBS), respectively. Horses were observed for any adverse reactions and the appearance of clinical manifestations of EVA twice daily for the first 2 weeks after immunization and once daily for an additional 2 weeks. Blood samples were collected in buffered sodium citrate (Monopt) tubes at 0, 2, 4, 6, 8, 10, 12, and 14 days postvaccination (DPV) for hematology. Blood...
samples were also collected for separation of peripheral blood mononuclear cells (PBMCs) for virus isolation at 0, 2, 4, 6, 8, 10, 12, 14, 21, 28, and 35 DPV. Serum samples were collected at 0, 2, 4, 6, 8, 10, 12, 14, 21, 28, and 35 DPV for the detection of neutralizing antibodies to the virus. Nasopharyngeal swabs were collected at 0, 2, 4, 6, 8, 10, 12, 14, 21, 28, and 35 DPV for virus isolation.

**Experimental challenge of horses with a heterologous strain of EAV and sample collection.** At 35 DPV, two groups of horses were intranasally challenged with 1.0 × 10⁷ PFU of heterologous EAV KY84 strain that was delivered in 5.0 ml of EMEM using a fenestrated catheter. Horses were monitored twice daily for 4 weeks for clinical manifestations of EVA. Blood for hematology (in buffered sodium citrate [Monojet]) was collected from all horses at 0, 2, 4, 6, 8, 10, 12, and 14 days postinfection (DPI) for complete blood cell counts. Nasopharyngeal swabs and blood samples were collected at 0, 2, 4, 6, 8, 10, 12, 14, 21, 28, and 35 DPI for virus isolation and neutralizing antibody determination.

**Clinical laboratory assays.** Hematological analysis was performed at the Hagyard Equine Medical Institute (4250 Iron Works Pike, Lexington, KY) using an electronic cell counter (Coulter Electronic Inc.). Differential counts were performed manually.

**Virus isolation.** Virus isolation was attempted from peripheral blood mononuclear cells (PBMCs) and nasal swabs using RK-13 cells as previously described (9). The RK-13 KY cells were inoculated with serial 10-fold dilutions (10⁶ to 10⁷) of each sample and overlaid with RK-13 growth medium containing 0.75% carbosyphil methyl cellulose. The cells were incubated at 37°C for 5 to 7 days, and plaques were visualized by staining of the monolayer with crystal violet. A second passage was performed on negative samples at 4 to 5 days after the initial passage. Virus isolates were confirmed as EAV by real-time RT-PCR (4, 20).

**Virus neutralization (VN) test.** The neutralizing antibody titers of the test sera were determined as described by Senne et al. (37, 40). Briefly, serial 2-fold dilutions of each sample from 1:4 to 1:10,248 were made in MEM (Invtrogen, Carlsbad, CA) containing 10% guinea pig complement (Rockland Immunoccheicals, Gilbertsville, PA). Each serum sample was tested in duplicate in 96-well plates. Equal volumes of a virus dilution containing an estimated 50% tissue infective dose (TCID₅₀) of 200 of the modified live virus vaccine strain of EAV (ARVAC, Fort Dodge Animal Health [now Pfizer Animal Health Inc. Kalamazoo, MI]) were added to each well, except the serum controls. The plates were shaken to ensure mixing of the well contents and then incubated for 1 h at 37°C. A suspension of RK-13 KY cells was added to each well in a volume double that of the serum-virus mixtures, and the plates were incubated for 72 h at 37°C, until viral cytopathic effect had fully developed in the virus control wells. The titer of a sample was recorded as the reciprocal of the highest serum dilution that provided at least 50% neutralization of the reference virus.

**Statistical analysis.** Statistical analysis was performed with the Student t test using Sigma Plot 11 (Systat Inc., Richmond, CA).

**GenBank accession numbers.** The nucleotide sequences of the full-length cDNA clones pEAVrMLV and pEAVrMLVB were deposited in GenBank under the accession numbers FJ798195 and FJ798196, respectively.

**RESULTS**

**Construction of full-length cDNA clones of the MLV vaccine strain (ARVAC) of EAV and determination of their infectivity.** A full-length cDNA clone of the MLV vaccine strain was assembled from four overlapping PCR fragments flanked by unique restriction sites. The obtained plasmid contained a bacteriophage T7 RNA polymerase promoter at the 5′ terminus of the viral genome, the 12,704-nucleotide full-length genome of EAV MLV, and a poly(A) tail of 20 residues at the 3′ end of the genome. Compared to the genome sequence of the parental MLV vaccine strain (ARVAC) of EAV (GenBank accession number EU586275) (53), the initially assembled full-length cDNA clone contained two nucleotide differences, 3289C→T (amino acid Ser1022→Leu in Nsp3) and 3933G→T (amino acid Ser1237→Ala in Nsp4), and this initial cDNA clone was named pEAVrMLV-S1022L&S1237A (Table 1). When IVT RNA synthesized from linearized pEAVrMLV-S1022L&S1237A plasmid containing 3289T and 3933G was electroporated into BHK-21 cells and EECs, it was found to be noninfectious. Subsequently, these two sites were reverted back to 3289C and 3933T using site-specific mutagenesis either individually or together. The resulting full-length infectious cDNA clones were identified as pEAVrMLV-S1237A, pEAVrMLV-S1022L, and pEAVrMLV (Table 1).

**Table 1. Nucleotide and amino acid differences between the parental MLV vaccine strain (ARVAC) and the full-length vaccine cDNA clones**

| Plasmid or virus name | Nt (amino acid) at position: | Restriction site BspEI | Genome replication | Infectious virus production |
|----------------------|-------------------------------|------------------------|-------------------|---------------------------|
| MLV parental virus   | C (Nsp3 Ser-1022) T (Nsp4 Ser-1237) C (N Arg-37) | No Yes Yes | Yes | Yes |
| pEAVrMLV-S1022L      | T (Nsp3 Leu-1022) G (Nsp4 Ala-1237) C (N Arg-37) | No No No | No | No |
| pEAVrMLV-S1237A      | T (Nsp3 Leu-1022) G (Nsp4 Ala-1237) C (N Arg-37) | No No No | Yes | Yes |
| pEAVrMLV             | C (Nsp3 Ser-1022) T (Nsp4 Ser-1237) C (N Arg-37) | No Yes Yes | Yes | Yes |
| pEAVrMLVB            | C (Nsp3 Ser-1022) T (Nsp4 Ser-1237) G (N Arg-37) | Yes Yes Yes | Yes | Yes |

**The in vitro-transcribed (IVT) full-length viral RNAs from the XhoI-linearized plasmids pEAVrMLV-S1022L&S1237A, pEAVrMLV-S1022L, pEAVrMLV-S1237A, and pEAVrMLV were then transfected into BHK-21 cells and EECs. At 24 h posttransfection, cells were examined by immunofluorescence assays using MAbs specific to the N protein and Nsp1 of EAV. As shown in Fig. 1, viral protein expressions were observed in cells transfected with pEAVrMLV-S1237A (panels g and h) or pEAVrMLV (i and j) as well as cells infected with the parental MLV vaccine strain (m and n) but were not observed in mock-transfected cells (a and b) and cells transfected with pEAVrMLV-S1022L&S1237A (c and d) or pEAVrMLV-S1022L (e and f). This showed that the reversion of 3933G→T alone did not make the IVT RNA infectious. In contrast, a clone with 3289T→C reversion alone or reversion of both substitutions (3289T→C and 3933G→T) resulted in infectious IVT RNA that led to complete CPE typical of EAV. When supernatants from the transfected cells were passaged into fresh BHK-21 cells and EECs after 96 h posttransfection, viral protein expressions and virus-specific CPE were observed in cells inoculated with supernatants from pEAVrMLV-S1237A- and pEAVrMLV-transfected cells but were not observed in cells inoculated with supernatants from pEAVrMLV-S1022L&S1237A- and pEAVrMLV-S1022L-transfected cells (data not shown). Taken together, these data demonstrated that the clones pEAVrMLV-S1022L&S1237A and pEAVrMLV-S1022L were noninfectious (no genomic replication and infectious progeny virus production), whereas the clones pEAVrMLV-S1237A and pEAVrMLV were infectious (with genomic replication and infecti-
tious progeny virus production; Table 1). The mutation on 3289C→T (Nsp3 Ser1022→Leu) is lethal, while the mutation on 3933T→G (Nsp4 Ser1237→Ala) is not critical for genomic replication and infectious virus production.

Full-length genomic sequencing of the recombinant viruses harvested from the transfected EECs (P0 virus) revealed that the cloned vaccine virus rMLV derived from the cDNA clone pEAVrMLV (GenBank accession number FJ798195) has 100% nucleotide identity to the previously published master sequence of the parental MLV vaccine strain of EAV (GenBank accession number EU586275). In order to distinguish the rMLV strain from the parental MLV vaccine strain, a silent point mutation was introduced into ORF7 (nucleotide 12423C→G encoding N Arg-37) of the pEAVrMLV cDNA clone to create a unique restriction site, BspEI, thus obtaining the full-length cDNA clone pEAVrMLVB (GenBank accession number FJ798196). Upon transfection into BHK-21 cells and EECs, genomic replication was observed (Fig. 1k and l), and infectious progeny virus was obtained from pEAVrMLVB-IVT RNA-transfected cells. Full-length genomic sequencing confirmed that the cloned vaccine virus rMLVB (P0) had a nucleotide sequence identical to the parental MLV vaccine virus except for the single nucleotide substitution at nucleotide 12423; the cloned vaccine virus rMLVB was genetically stable after 10 passages in BHK-21 cells. A 710-bp RT-PCR fragment amplified from nucleotides 11893 to 12603 was cleaved by BspEI in the cloned vaccine virus rMLVB (P0, P5, and P10) but was not cleaved by BspEI in the parental MLV vaccine virus and the recombinant rMLV virus (Fig. 2). These clearly demonstrated that the single nucleotide substitution is stable during serial passage in cell culture.

Allelic discrimination real-time RT-PCR assay. The parental MLV vaccine strain as well as all of the field isolates of EAV had a conserved nucleotide C at position 12423, whereas the cloned vaccine virus rMLVB had a G at this position; therefore, this was used as a unique genetic marker to distinguish the cloned vaccine virus rMLVB from the parental MLV vaccine strain and field isolates of EAV. While the BspEI cleavage assay based on this unique genetic marker at position 12423 is able to distinguish the cloned vaccine virus rMLVB from field isolates of EAV, the assay is laborious and time-consuming. Accordingly, an allelic discrimination real-time RT-PCR assay based on the single nucleotide difference at position 12423 was developed to fulfill this need. The parental MLV vaccine strain (ARVAC) was recognized by probe C but not by probe G (Fig. 3a); in contrast, the cloned vaccine virus rMLVB was recognized by probe G but not by probe C (Fig. 3b). Further analysis of 60 field and laboratory strains of EAV indicated that these

FIG 1 Results of IFA testing of BHK-21 cells mock transfected or transfected with IVT viral RNA from various EAV cDNA clones as well as cells infected with the parental vaccine strain of EAV. At 24 h posttransfection or postinfection, cells were examined by IFA using MAb 12A4 against EAV Nsp1 protein (α nsp1) and MAb 3E2 against EAV N protein (α N) followed by Texas Red-conjugated goat anti-mouse immunoglobulin (red color). Cell nucleus was stained by Hoechst 33342 (blue color).
strains were also recognized only by probe C but not by probe G (Fig. 3c). Negative control samples were neither recognized by probe C nor by probe G (Fig. 3d).

In vitro growth of the cloned vaccine virus. The cloned rMLVB virus and the parental MLV vaccine virus had identical growth kinetics in either EECs or BHK21 cells (Fig. 4a). Titers peaked at 24 to 48 hpi for both viruses, with maximal titers of \( \approx 4 \times 10^8 \) PFU/ml. Plaque morphology of these two viruses was also determined, and the plaque size produced by the cloned rMLVB virus was very similar to that of the parental MLV (Fig. 4b). These results indicate that the cloned vaccine virus rMLVB possesses in vitro properties similar to those of the parental MLV vaccine virus.

Safety and the immunogenicity of the rMLVB vaccine strain of EAV. Eight EAV seronegative horses were randomly assigned into two groups and inoculated intramuscularly with PBS or rMLVB virus, respectively (PBS control group one, \( n = 2 \) horses; rMLVB virus group two, \( n = 6 \) horses). Experimentally vaccinated horses were monitored up to 35 days postvaccination. All horses inoculated with PBS or rMLVB virus remained healthy and did not show any adverse effects after vaccination. The rectal temperature of all horses remained within the normal range of 36.6 to 38.0°C up to 35 days postvaccination (Fig. 5a). Results of virus isolation from PBMCs and nasal swabs after vaccination are summarized in Table 2. No virus was isolated from PBMCs and nasal

![FIG 2](image_url)

Differentiation between cloned viruses and the parental EAV MLV vaccine strain. A BspEI restriction site was introduced into the full-length cDNA clone for distinguishing the cloned virus rMLVB from the parental MLV virus. A 710-bp RT-PCR fragment containing the BspEI site was amplified and then digested with BspEI. The presence of a BspEI site resulted in fragments of 530 bp and 180 bp after digestion with BspEI.

![FIG 3](image_url)

An allelic discrimination real-time RT-PCR assay for distinguishing cloned virus rMLVB from parental MLV vaccine virus as well as field and laboratory strains of EAV.
The safety and efficacy of the rMLVB vaccine were evaluated by experimental vaccination and challenge with the heterologous KY84 field strain of EAV. The serologic response of the horses vaccinated with the commercial vaccine virus as previously described. When electroporated, IVT RNA from a first assembled full-length cDNA clone was noninfectious due to a single nonsynonymous nucleotide substitution in the Nsp3 coding region of ORF1a (resulting in an Ser1022→Leu substitution in Nsp3). It has been previously shown that arterivirus Nsp3 plays a critical role in membrane modification and double membrane vesicle (DMV) formation during the early virus replication cycle (12, 39, 43). Substitutions of each of a cluster of four conserved cysteine residues, residing in a predicted luminal loop of Nsp3, completely blocked DMV formation. Some of these mutant Nsp3 proteins were also found to be highly cytotoxic, in particular, by exerting a dramatic effect on the endoplasmic reticulum. Furthermore, it has been reported that these cysteine mutants also affect the pp1a autoprocessing. Interestingly, the lethal mutation (Ser1022→Leu) in our infectious cDNA clone was located in the cytoplasmic tail of the Nsp3 protein; currently, it is not clear how this mutation adversely affected replication of the virus. Once this lethal mutation was removed and transcripts from the new full-length clone pEAVrMLVB were electroporated, CPE typical of EAV infection supervened. Subsequently, a transcriptionally silent marker mutation was introduced into the ORF7 region of pEAVrMLVB, thereby creating a unique BspEI restriction site. The clone carrying this genetic marker, pEAVMLVB, was used for additional in vitro and in vivo studies. The companion allelic discrimination rRT-PCR that was developed allows for differentiation of the recombinant MLV vaccine from field strains of EAV and facilitates molecular epidemiological investigations of reported vaccine-associated outbreaks of EVA. Stability of the recombinant virus was further established by sequential cell culture passage of the virus, confirming presence of the unique BspEI restriction site following 10 serial passages. In summary, the infectious cDNA clone was noninfectious due to a single nonsynonymous nucleotide substitution in the Nsp3 coding region of ORF1a (resulting in an Ser1022→Leu substitution in Nsp3). It has been previously shown that arterivirus Nsp3 plays a critical role in membrane modification and double membrane vesicle (DMV) formation during the early virus replication cycle (12, 39, 43). Substitutions of each of a cluster of four conserved cysteine residues, residing in a predicted luminal loop of Nsp3, completely blocked DMV formation. Some of these mutant Nsp3 proteins were also found to be highly cytotoxic, in particular, by exerting a dramatic effect on the endoplasmic reticulum. Furthermore, it has been reported that these cysteine mutants also affect the pp1a autoprocessing. Interestingly, the lethal mutation (Ser1022→Leu) in our infectious cDNA clone was located in the cytoplasmic tail of the Nsp3 protein; currently, it is not clear how this mutation adversely affected replication of the virus. Once this lethal mutation was removed and transcripts from the new full-length clone pEAVrMLVB were electroporated, CPE typical of EAV infection supervened. Subsequently, a transcriptionally silent marker mutation was introduced into the ORF7 region of pEAVrMLVB, thereby creating a unique BspEI restriction site. The clone carrying this genetic marker, pEAVMLVB, was used for additional in vitro and in vivo studies. The companion allelic discrimination rRT-PCR that was developed allows for differentiation of the recombinant MLV vaccine from field strains of EAV and facilitates molecular epidemiological investigations of reported vaccine-associated outbreaks of EVA. Stability of the recombinant virus was further established by sequential cell culture passage of the virus, confirming presence of the unique BspEI restriction site following 10 serial passages. In summary, the infectious cDNA clone derived from the current MLV vaccine strain is identical to the wild-type virus with the exception of the single nucleotide substitution introduced at position 12423 in ORF7. The recombinant vaccine virus has the same in vitro phenotypic properties as the parental commercial MLV vaccine.

The safety and efficacy of the rMLVB vaccine were evaluated by experimental vaccination and challenge with the heterologous KY84 field strain of EAV. The serologic response of the horses vaccinated with rMLVB was identical to that observed in horses vaccinated with the commercial vaccine virus as previously de-
scribed (44, 45). There was no adverse effect on the safety and induction of the humoral immune response resulting from incorporation of a single noncoding (synonymous) nucleic acid substitution in ORF7. Similar to the unmodified MLV vaccine studies (44, 50), a very small amount of infectious virus (<2 PFU/ml) was transiently detected in nasal secretions and in the PBMCs of the vaccinated horses within the first week after immunization. Collectively, these data prove that the recombinant virus is very similar, if not identical, to the unmodified commercial MLV vaccine virus in regard to virus shedding pattern and its ability to stimulate a humoral antibody response. Compared to the control animals, the horses vaccinated with rMLVB developed significantly reduced levels of viremia and shorter duration of virus shedding after challenge with the heterologous EAV KY84 strain. However, the level of protection afforded by vaccination did not completely protect horses from developing clinical signs of EVA following challenge with this heterologous strain. While it is believed there is only one known serotype of EAV, field strains differ in their neutralization phenotype (1, 6, 7, 21, 33, 56, 57). With the exception of a few EAV field strains (2, 7, 56), the majority of the field strains are neutralized to a higher titer (1:64 to 1:1,024) by polyclonal antiserum raised against the virulent Bucyrus strain of EAV (EAV VBS). In contrast, it has been shown previously that the serum from horses vaccinated with the MLV vaccine strain neutralizes some EAV field strains to a lower titer (1:8 to 1:64) (1, 7, 16, 56); this includes the EAV KY84 strain used in this study. This may explain why horses were not completely protected from developing clinical signs of EAV following challenge with the heterologous EAV KY84 strain. Furthermore, equine antiserum against the prototype EAV VBS neutralizes the EAV KY84 to a higher titer (1:64 to 1:256) (1, 7, 56), and horses vaccinated with a subunit vaccine containing the GP5 and M heterodimer of the EAV VBS were well protected when challenged with the same KY84 strain of EAV (3). This further confirms the importance of high-titer neutralizing antibodies (≥1:64) in protecting against the clinical signs of EAV infection (14, 50). It is also possible that the very high challenge virus dose (1.0 × 10⁷ PFU) used in this study increased the likelihood of a clinical response in the vaccinated horses. It should be emphasized that the commercial MLV vaccine had been successfully used to control several major EVA outbreaks in the United States in the past 20 to 25 years (24, 30, 46, 48–50). These data also emphasize the importance of conducting additional in-depth cross-neutralization studies using more recent EAV isolates representing all three phylogenetic clades of EAV (North American and two European [EU-1 and EU-2]). It remains to be seen whether more heterologous challenge studies of horses vaccinated with the commercial MLV are indicated. The infectious cDNA clone of the

FIG 5 Experimental vaccination and challenge of horses. Group one (n = 2) and group two (n = 6) horses were intramuscularly inoculated with PBS or vaccinated with the cloned rMLVB virus (1 × 10⁷ PFU), respectively. At 35 days postvaccination, all horses were challenged with a heterologous EAV KY84 strain (1 × 10⁶ PFU). (a) Rectal temperature after vaccination and challenge; (b) neutralizing antibody titers to EAV after vaccination and challenge; (c) virus isolation results from nasal swabs after challenge; (d) virus isolation results from PBMCs after challenge; viral loads with significant difference are indicated by the asterisk.
MLV vaccine strain of EAV described in this study 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.

In summary, we have generated an infectious cDNA clone of the current MLV vaccine strain with a unique nucleotide mutation in ORF7 to distinguish recombinant vaccine virus from laboratory and field strains of EAV using a companion real-time RT-PCR assay. The vaccine clone pEAVrMLVB could be further genetically manipulated to develop a marker vaccine by either deleting an immunogenic viral epitope(s) to create a negative marker or by inserting a foreign antigenic epitope(s) to create a positive marker. Companion serological assays can be developed to detect antibody responses to the deleted immunogenic viral epitope(s) or to the inserted foreign antigenic epitope(s). Such a marker vaccine would provide a means of serologically differentiating EAV naturally infected from vaccinated animals by using the companion serological assays. Furthermore, this infectious cDNA clone could be used to design and develop a rational, broadly protective recombinant vaccine(s) by genetically engineering the immunodominant neutralizing epitopes from EAV strains that differ in their neutralization phenotypes. We previously developed an infectious cDNA clone (pEAVrVBS) from the virulent Bucyrus strain of EAV (8). The virulent pEAVrVBS clone and the attenuated vaccine clone pEAVrMLVB provide valuable research tools to study the life cycle of the virus (attachment, entry, replication, transcription, translation, and assembly) and viral pathogenesis, as well as the immune response to the virus.

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**TABLE 2 Results of attempts to isolate virus in RK-13 cells from nasal swab and PBMCs of horses after experimental vaccination with PBS or rMLVB virus**

| Horse ID | Days postvaccination* | Specimen | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 |
|----------|------------------------|----------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C7       | PBS                    | Nasal swab | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| C11      | PBS                    | Nasal swab | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| C12      | rMLVB                  | Nasal swab | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| C17      | rMLVB                  | Nasal swab | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| C19      | rMLVB                  | Nasal swab | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| C24      | rMLVB                  | Nasal swab | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| D2       | rMLVB                  | Nasal swab | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| D8       | rMLVB                  | Nasal swab | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |

a –, virus isolation negative; +, virus isolated only after second passage in cell culture.
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