Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Experimental infection of horses with Madariaga virus and initial evaluation of equine-to-mosquito transmission

Loni Taylor
Texas A&M University
Andre Correa
Texas A&M University
Wendy Tang
Texas A&M University
William Sames IV
Independent Researcher
Gabriel Hamer
Texas A&M University
Lauro Velazquez-Salinas
Texas A&M University
Tereza Magalhaes
tereza.magalhaes@ag.tamu.edu
Texas A&M University

Research Article

Keywords: Alphavirus madariaga, eastern equine encephalitis virus, alphavirus, equine encephalitides, amplifying host, mosquito transmission, high-containment, vector-borne disease

Posted Date: October 6th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11012753/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Abstract

Background

Madariaga virus (MADV) is an understudied mosquito-borne alphavirus that circulates widely in Latin America. Following spillover from its enzootic cycle, MADV can infect both equids and humans, with equine cases often preceding human infections. Despite the high equine morbidity and case fatality rates reported during epizootics, it remains unclear whether infected horses can contribute to virus amplification by serving as a source of infection for mosquitoes. We sought to establish a large-animal experimental system for studying MADV infection and equine-to-mosquito transmission under high-containment conditions.

Methods

Two pilot trials were conducted. Trial 1 evaluated the feasibility of housing horses and conducting direct mosquito-feeding under containment. In Trial 2, two horses were inoculated subcutaneously with MADV and monitored for 14 days to characterize clinical, laboratory, virological, and serological responses, with histopathological analysis performed after euthanasia. Two mosquito species were allowed to feed directly on the horses during the first 8 days post-inoculation and were subsequently tested for MADV RNA.

Results

Trial 1 demonstrated the feasibility of horse housing and direct mosquito-feeding under containment. In Trial 2, conducted in the high-containment spaces, neither horse developed overt clinical disease, although one exhibited delayed hindlimb proprioception for multiple days. MADV RNA was not detected in horse plasma or mosquitoes that fed on inoculated horses. Both horses seroconverted, with one developing a stronger neutralizing antibody response. Hematological and biochemical parameters remained within reference intervals. No gross or histopathological central nervous system lesions were observed.

Conclusions

These pilot studies establish the feasibility of a large-animal BSL-3 platform for experimental MADV infection and direct equine-to-mosquito transmission studies and provide an initial experimental evaluation of whether infected horses can serve as a source of MADV for mosquito infection. Importantly, this system can be expanded to include mosquito-to-horse transmission. This would allow both directions of the mosquito-equid transmission cycle to be reproduced experimentally, enabling studies of transmission determinants and pathogenesis, as well as evaluation of vaccine efficacy,

durability of immunity, and therapeutic interventions. The platform could be applied to MADV and other mosquito-borne arboviruses of human and animal health importance that infect equids.

1. Background

New World equine encephalitic alphaviruses include members of the eastern equine encephalitis virus (EEEV), western equine encephalitis virus (WEEV), and Venezuelan equine encephalitis virus (VEEV) complexes (1–3). These mosquito-borne viruses are maintained in enzootic cycles involving vertebrate hosts and can spill over to infect equids and humans, with outcomes ranging from asymptomatic infection to severe neurological disease and death (4–8).

Following spillover, the role of infected horses in the transmission cycle varies among these viruses, and the distinction between dead-end and amplifying hosts is not always clear. Experimental infection studies have shown that horses infected with North American EEEV (NA EEEV) develop transient viremia at levels usually insufficient to infect mosquitoes and are therefore generally considered dead-end hosts (9, 10). However, NA EEEV horse-to-mosquito transmission has been demonstrated under certain experimental conditions (10, 11). For VEEV, subtypes IAB and IC, also named ‘epizootic’ VEEV strains, can produce high viremia in horses sufficient to infect mosquitoes, leading to an equid amplification cycle during epizootics (12–15). When equine amplification of alphaviruses occurs, infected horses provide a source of virus for mosquito infection and can contribute to transmission among horses and to humans, potentially increasing the extent of an outbreak, as demonstrated during the 1969–1971 VEEV epizootics in Latin America and North America (4, 16). Equine-amplifying VEEV strains are thought to have emerged from ancestral enzootic strains through minor genetic changes (17, 18).

Madariaga virus (MADV; *Alphavirus madariaga*) is an understudied member of the EEEV complex that is widely distributed throughout Latin America (2, 19). Severe human disease was historically considered rare, but an outbreak of human encephalitis in Panama in 2010, which occurred concurrently with an equine epizootic, challenged this idea (5). Equine epizootics have been reported in at least six Latin American countries, with reported morbidity rates ranging from 17% to 60% and case fatality rates from 40% to 98% (19–21). Yet, whether these epizootics result solely from spillover or also involve amplification by horses remains unknown, representing an important gap in understanding MADV transmission at the animal-to-vector-to-human interface.

Much of what is known about the role of horses in alphavirus transmission comes from experimental studies conducted decades ago. Horses were infected with different virus strains by needle inoculation or the bite of infected mosquitoes, and their potential to serve as amplifying hosts was assessed by measuring viremia or by allowing mosquitoes to feed directly on infected animals (10, 12–15). Similar experimental approaches have been used to investigate the role of horses in the transmission of West Nile virus (22). Conducting such studies requires specialized infrastructure for housing infected large animals and maintaining infected mosquito vectors under high-containment conditions, as well as a multidisciplinary team with expertise in vector biology, arbovirology, and equine medicine. Experimental

infection of horses, clinical monitoring and sample collection, mosquito feeding, and subsequent mosquito maintenance and manipulation must therefore be planned and performed within current biosafety, biocontainment, and animal research requirements.

Here, we conducted two trials to establish an experimental system for MADV infection and mosquito-feeding studies in horses under biosafety level 3 (BSL-3) containment. To our knowledge, the last comparable experimental horse-mosquito study was conducted more than two decades ago, and no such study had previously been conducted at our institution. The first trial was designed to determine whether horses could be housed under containment and undergo repeated direct mosquito-feeding procedures without adverse effects on animal welfare, while also allowing optimization of the experimental procedures before virus infection. Trial 2 proceeded only after successful completion of Trial 1 and consisted of a pilot infection study in which two horses were experimentally infected with MADV to characterize the clinical course and virological features of infection and evaluate whether mosquitoes could acquire the virus after feeding on infected horses. In addition to these objectives, the large-animal-arthropod high-containment system established here can support a range of experimental studies, including investigations of transmission determinants, pathogenesis, and immune responses, as well as the evaluation of preventive and therapeutic interventions.

2. Methods

2.1 Virus and mosquitoes

The virus used in this study was MADV strain 165 (MADV-BR; GenBank accession no. MZ389692.1), isolated by our group from the central nervous system (CNS) of a horse that died of neurological disease in 2019 in Northeast Brazil (23). A previously prepared passage 11 (P11) stock propagated in Vero cells (ATCC CCL-81) was used for horse inoculation. The stock titer was $1.2x10^7$ plaque forming units (PFU)/mL and free of mycoplasma.

Aedes taeniorhynchus (F0) and *Aedes aegypti* (F5) were used in the experiments. *Aedes taeniorhynchus* were collected as larvae in Brazoria County, Texas, in September 2025 using a dipper. Larvae were transported to the walk-in insectary at Texas A&M University (TAMU) and reared to adults under standard conditions (70% relative humidity, 28ºC, and a 12:12 h light:dark cycle). *Aedes aegypti* were collected as eggs using ovitraps in Hidalgo County, Texas, in September 2023. Eggs were transported to the TAMU insectary, and mosquitoes were reared under the same conditions described above. F5 *Ae. aegypti* were used in the experiments. These species were selected because our group has previously demonstrated their vector competence for MADV (24). In addition, *Ae. taeniorhynchus* has also been implicated as a putative epizootic vector of the virus (25, 26).

2.2 Mosquito preparation for feeding

For both mosquito species, 50–80 females, 5–7 days post-emergence, were placed per 480-mL carton and transferred to the arthropod containment level 2 (ACL-2) (Trial 1) or ACL-3 (Trial 2) laboratories

within the biosafety facility two days before the experiments for acclimation. Mosquitoes were maintained in growth chambers at 28ºC and 70% relative humidity under a 12:12 h light:dark cycle. Prior to feeding on horses, females were deprived of sugar for 48 h but maintained with access to water.

2.3 Trial 1 – Assessment of horse welfare and experimental feasibility

As required by the Institutional Animal Care and Use Committee (IACUC) and Biosafety Office before proceeding with the infection trial, the first trial, conducted at the large-animal facility, was designed to evaluate horse welfare and behavior during confinement and daily mosquito-feeding procedures and to identify any modifications to the experimental protocol that might be needed. Two mares (Quarter Horse; 18 and 20 years of age) with no known history of prior vaccination against NA EEEV, WEEV and VEEV were obtained from commercial vendors and tested for antibodies against NA EEEV, WEEV, and VEEV using plaque reduction neutralization test (PRNT), at the United States Department of Agriculture (USDA) National Veterinary Services Laboratory (NVSL) in Ames, IA. One horse had a VEEV $PRNT_{80}$ titer of 1:100, whereas the second horse was seronegative for all viruses tested.

Horses were transferred to the mock BSL-3 agriculture (BSL-3Ag) facility, a training space with stalls and infrastructure similar to those of the BSL-3Ag facility. Horses were housed individually in 15x13 foot stalls within the same room and allowed to acclimate for 48 h before initiation of mosquito-feeding procedures. To support animal welfare and refinement of the experimental model, horses were housed on deep kiln-dried pine shavings and provided free-choice coastal Bermuda hay in slow-feeder hay nets and a complete pelleted horse feed. Additional enrichment and husbandry measures included daily treats, continuous auditory enrichment via radio, daily grooming, and twice-daily stall cleaning. These measures were implemented to promote normal behavior and minimize stress associated with confinement.

Mosquito cartons were then brought to the stalls and secured to the horses' necks with rolled gauze (Fig. 1), and mosquitoes were allowed to feed for 30 min while the horses were provided pelleted feed and/or alfalfa leaves. Following feeding, mosquito cartons were transferred back to the ACL-2 laboratory. Mosquito feeding was performed daily for three consecutive days in the morning, alternating between sides of the horse's neck on successive days. Mosquito-feeding sites on the horses' necks were inspected daily for local reactions.

Blood samples were collected daily from the horses for biochemical analyses to identify potential physiological changes associated with confinement and experimental procedures. Clinical evaluations were performed twice daily.

Horse behavior and results of the hematological and biochemical analyses were evaluated by the research team and members of the IACUC and Biosafety teams. Horses tolerated confinement and the mosquito-feeding procedures well, and no atypical changes were detected in the hematological and

biochemical parameters evaluated. Local reactions to mosquito bites were minimal when present and resolved without intervention within 24 h.

2.4 Trial 2 – Experimental infection of horses with MADV

2.4.1 Virus inoculation and mosquito feeding

Following IACUC and Biosafety approval of the procedures evaluated in Trial 1, a second trial was conducted in the BSL-3Ag facility. Horses were housed and cared for as described for Trial 1. Two mares (Quarter Horses; both 20 years of age), including the horse from Trial 1 that tested negative for antibodies against NA EEEV, WEEV, and VEEV (Horse 1), were transferred to the BSL-3Ag containment facility and allowed to acclimate for 72 h before virus inoculation. For Horse 2, vaccination records for the preceding three years indicated no vaccination against NA EEEV, WEEV, or VEEV. The horse tested negative for antibodies against WEEV and VEEV but had an NA EEEV-neutralizing antibody titer of 1:10. On Day 0, both horses were inoculated subcutaneously in the low cervical region with 4,000 plaque-forming units (PFU) of MADV-BR (P11) in 200uL of plain cell culture medium using a tuberculin syringe.

Beginning on Day 1 post-inoculation, one carton containing *Ae. taeniorhynchus* was secured to each horse's neck with rolled gauze for 30 min to allow mosquito feeding. The procedure was repeated on Day 2. Beginning on Day 3, an additional carton containing *Ae. aegypti* was placed on each horse and allowed to feed concurrently with *Ae. taeniorhynchus*. Mosquito feeding was performed daily through Day 8 post-inoculation, alternating between sides of the horse's neck on successive days. After feeding, mosquito cartons were transferred back to the ACL-3 laboratory, where bloodfed females were sorted from unengorged females and maintained for 21 days under standard rearing conditions with access to water and 10% sucrose. At the end of the 21-day period, mosquitoes were cold-killed, pooled by species and feeding date, with up to 50 mosquitoes per pool, and stored at -80ºC. Mosquito pools were placed in tubes containing 3–5 borosilicate beads and PBS supplemented with 20% fetal bovine serum at a volume of 50 µL per mosquito.

Horses were monitored for 14 days post-inoculation (dpi). Blood samples were collected daily from Day 0 through Day 14. To assess potential viral shedding, nasal and fecal samples were collected daily from Days 2 through 7. Nasal sampling consisted of placing sterile filter paper near the horses' nares for the duration of the mosquito-feeding period and collection of nasal swabs. Fecal samples were collected using a scoop. Collected samples were kept at -80ºC.

The animals were humanely euthanized at 14 dpi using approved methods from the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals. Horses were sedated with 300 mg of xylazine administered IV followed by 120 mL of pentobarbital administered IV. Confirmation of euthanasia was conducted via the absence of a heartbeat and corneal reflex. Necropsy was then performed for tissue collection.

2.4.2 Clinical monitoring

Horses underwent thorough clinical evaluations beginning upon arrival in the biocontainment stalls and continuing throughout the study. Physical and neurologic exams were performed twice daily, once in the morning and once in the afternoon. These examinations included measurement of rectal temperature, heart rate, and respiratory rate; assessment for depression, anorexia, and lethargy; and neurologic evaluation for weakness, ataxia, proprioceptive deficits, seizure activity, cranial nerve deficits, and paralysis. Neurologic abnormalities were graded using the University of California, Davis, neurologic examination protocol (27) and the Mayhew ataxia grading system (grades 0–5).

2.4.3 Hematology and blood biochemistry

Blood samples were collected prior to MADV inoculation and daily until Day 14 post- inoculation. Complete blood counts and plasma biochemical analyses were performed using a VetScan HM5 hematology analyzer and VetScan VS2 chemistry analyzer, respectively (Zoetis, Parsippany, NJ, USA). Hematological parameters included total white blood cell, lymphocyte, monocyte, neutrophil, eosinophil, basophil, red blood cell, platelet counts, hemoglobin concentration, hematocrit, and red blood cell and platelet indices. Plasma biochemical parameters included albumin, aspartate aminotransferase, blood urea nitrogen, calcium, chloride, creatine kinase, creatinine, gamma-glutamyl transferase, glucose, total bilirubin, total protein, potassium, sodium, total carbon dioxide, and globulin. Hematological and biochemical values were evaluated relative to the reference intervals provided by the respective analyzers.

2.4.4 Neutralizing antibodies

Sera collected before virus inoculation and at 10 and 14 dpi were evaluated for MADV-neutralizing antibodies by PRNT as previously described (28, under review). Briefly, serum samples were heat-inactivated at 56ºC for 30 min, serially diluted from 1:10 to 1:640, and mixed 1:1 with a 1:30,000 dilution of the MADV-BR stock used for horse inoculation. Serum-virus mixtures were incubated for 1h at 37ºC with 5% CO_2, inoculated onto 90–100% confluent CCL-81 Vero cells (ATCC) in 6-well plates and adsorbed for 1 h at 37ºC with 5% CO_2. The control was MADV-BR mixed 1:1 with M199 medium (Thermo Fisher Scientific, Waltham, MA, USA). Following adsorption, cells were overlaid with 3 mL of 0.5% agarose and incubated for 48 h, followed by a second agarose overlay containing 4% neutral red. Plaques were counted after an additional 24 h, and plaque reduction was calculated relative to the virus control. $PRNT_{50}$ titers were defined as the reciprocal of the highest serum dilution resulting in ≥ 50% plaque reduction.

2.4.5 RNAemia and mosquito infection rates

To assess MADV RNAemia, viral RNA was extracted from 50 µL of horse plasma using the Mag-Bind DNA/RNA 96 Kit (Omega Bio-tek, Inc., Norcross, GA, USA) and the KingFisher Flex System (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturers' instructions. RNA was eluted in 50 µL of water.

MADV RNA presence in plasma was assessed by reverse transcription quantitative PCR (RT-qPCR) using 8 µL of RNA in a 20-µL reaction with the iTaq Universal One-Step RT-qPCR kit (Bio-Rad Laboratories, Hercules, CA, USA). Primers and probe targeting MADV nsP2 (24) were: forward, 5'-GGCTGAACAGGTGCTAGTTAT-3'; reverse, 5'-CTATTCCAATCCCGGACTTTCA-3'; and probe, 5'-CGCGCCGGTAGGTACAAAGTAGAA-3' (6-FAM/ZEN/3' IB FQ). Final primer and probe concentrations were 400 nM and 250 nM, respectively, and the amplicon size was 117 bp. Reactions were performed on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) at 50ºC for 10 min and 95ºC for 2 min, followed by 40 cycles of 95ºC for 15 s and 60ºC for 30 s. Samples with cycle threshold (Ct) values ≤ 39 were considered positive. The assay limit of detection, determined using an in vitro-purified transcript, was approximately 8 RNA copies per reaction.

Previously stored mosquito pools were homogenized for 1 min at 34 Hz using a TissueLyser II (Qiagen, Germantown, MD, USA) and centrifuged at 14,000 x g for 5 min at 4ºC. Viral RNA was extracted from 50 µL of supernatant and tested for MADV RNA by RT-qPCR as described above. Mosquito infection rates were estimated from pooled samples using the minimum infection rate (MIR), calculated as the number of MADV RNA-positive pools divided by the total number of mosquitoes tested and expressed as a percentage.

2.4.6 Pathology

Organ samples, including brain, spinal cord, trigeminal and celiac-mesenteric ganglia, visceral organs (heart, spleen, liver, kidney and lungs) were collected collected at necropsy and fixed in 10% neutral-buffered formalin. Tissues were subsequently paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E) using standard procedures. For a comprehensive histologic analysis of the central nervous system, the following areas of the brain and spinal cord were sectioned: frontal lobe, parietal lobe, basal nuclei, hippocampus, midbrain, pons, medulla oblongata, cerebellum, cervical, thoracic, and lumbar spinal cord and cauda equina. Slides were reviewed by a board-certified veterinary pathologist.

2.4.7 E2 sequencing

Because mutations in the alphavirus E2 envelope glycoprotein have been associated with changes in equine virulence (17, 18), and because cell-culture passage can lead to mutations in E2 (29), we sequenced the E2 gene of the MADV-BR P11 stock used for horse inoculation and compared it with the original P5 sequence. The P5 sequence deposited in GenBank (accession no. MZ389692.1) was used as the reference sequence and for primer design. The following primers were designed to amplify an approximately 1.2-kb region spanning the entire MADV E2 gene: forward (5'-3') – AGTGCAATAGCAGACGGACC; reverse (5'-3') – ACACAGTAAGCAGCGTAGCA.

Viral RNA from the P11 MADV-BR stock was reverse-transcribed using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA), with the MADV-BR -specific reverse primer listed above. The cDNA was then amplified by PCR using the AccuPrime Pfx SuperMix (Thermo Fisher Scientific, Waltham, MA, USA) and the MADV-BR-specific forward and reverse primers listed above. The

PCR amplicon was purified using the QIAquick Gel Extraction Kit (QIAGEN, Germantown, MD, USA) and submitted for Sanger sequencing using the same forward and reverse primers used for PCR amplification. Sequencing chromatograms were imported into UGENE v53.0, and low-quality regions at the ends of the reads were trimmed following visual inspection. The resulting sequences were mapped to the original MADV-BR sequence deposited in GenBank (accession no. MZ389692.1), and a consensus sequence was generated. Nucleotide discrepancies were manually verified by inspection of the corresponding chromatograms. The consensus E2 sequence was aligned with the original MADB-BR P5 sequence using BioEdit to identify nucleotide and amino acid differences. In addition, the E2 amino acid sequence was compared with 75 additional MADV E2 sequences to determine whether amino acid variants identified in the P11 stock were present among other available MADV sequences.

3. Results

3.1 Clinical signs

Neither horse developed overt systemic or severe neurological disease following MADV challenge, and both remained alive throughout the 14-day observation period. Heart rate and respiratory rate remained stable throughout the study. No febrile temperatures were recorded, but Horse 1 showed mild increases in body temperature at 1 and 2 dpi, both during the afternoon measurements, reaching a maximum of 37.9ºC (100.2ºF).

The primary clinical abnormality observed was mild delayed proprioception in Horse 1, which was first observed at 1 dpi and persisted through 6 dpi (Fig. 2). The sign was not observed from 7 to 9 dpi but was noted again during one clinical evaluation at 10 dpi. Reduced anal tonus was observed in Horse 2 at 7 dpi. No other monitored clinical or neurological abnormalities were observed in either horse.

Local reactions to inoculation and mosquito feeding were mild and self-limiting. Horse 1 developed a localized plaque of edema at the inoculation site that increased in size from 1 to 3 dpi, began to resolve at 4 dpi, and had completely resolved by 5 dpi. The same horse also developed transient multifocal, edematous skin elevations consisting of ~ 5 mm papules the mosquito-feeding sites after each feeding, which was mostly resolved by the evening clinical evaluation and completely resolved within 24 h (Fig. 3). Horse 2 developed mild edema at the inoculation site, which resolved more rapidly than in Horse 1, and did not develop local reactions to mosquito bites.

3.2 Hematological and biochemical markers

Hematological parameters showed some variation over the study period, including a transient increase in total leukocyte, neutrophil, and monocyte counts in Horse 1 at 1 dpi (Fig. 4). However, these fluctuations remained within the respective reference intervals. Biochemical parameters likewise remained within their respective reference intervals throughout the study in both horses.

3.3 MADV RNA in horse plasma and mosquito pools

No MADV RNA was detected by RT-qPCR in horse plasma. Because no RNAemia was detected in either horse, nasal swabs, filter papers, and fecal samples collected to assess potential viral shedding were not tested. No mosquito pools tested positive for MADV RNA, corresponding to an MIR of 0%.

3.4 Neutralizing antibody response

Both horses seroconverted following MADV inoculation, with $PRNT_{50}$ titers increasing over time. Titers were higher in Horse 1 than in Horse 2 at both 10 and 14 dpi (Table 1).

Table 1
Neutralizing antibody response against Madariaga virus (MADV) in horses following experimental challenge with MADV (Trial 2).

Timepoint	**$PRNT_{50}$**	
	Horse 1	**Horse 2**
Pre-challenge	< 10	< 10
10 dpi	160	10
14 dpi	≥ 640	40

$PRNT_{50}$: 50% plaque reduction neutralization test titer; dpi: days post-inoculation. Values shown represent the reciprocal dilution leading to $PRNT_{50}$.

3.5 Pathology

No gross or histologic lesions were observed in all examined tissues from either horse.

3.6 E2 sequencing

Sequence analysis of the MADV-BR E2 gene identified two nucleotide substitutions in the P11 stock relative to the P5 reference sequence. One resulted in a nonsynonymous substitution at E2 residue 29, replacing leucine with arginine (L29R; CTG→CGG; genome position 9042). The other was a synonymous substitution at E2 residue 242, where asparagine was retained (N242N; AAT→AAC; genome position 9682). Arginine at E2 residue 29 was not observed among 75 additional MADV E2 sequences examined. The MADV-BR E2 sequence generated here is available as a supplementary material.

4. Discussion

The primary objective of this study was to establish and evaluate a BSL-3 experimental system for conducting MADV infection and mosquito-feeding studies in horses. Specifically, in Trial 1, we sought to determine whether horses could be safely maintained under biocontainment conditions and whether serial mosquito-feeding experiments could be performed within this setting; in Trial 2, we provided a preliminary assessment of whether experimentally infected horses could serve as a source of virus for

mosquito infection. Given the complexity of the experimental procedures, the biosafety and animal welfare considerations involved, and the fact that this type of study had not previously been conducted at our institution, the IACUC and Biosafety Office required successful completion of Trial 1 before the MADV infection study could proceed. Trial 1 was successfully completed, with no adverse effects on horse welfare associated with confinement or the mosquito-feeding procedures, allowing the study to proceed to Trial 2.

In Trial 2, two mares inoculated subcutaneously with MADV-BR, a strain isolated from a fatal equine case (23), did not develop overt clinical disease. Clinical signs in equids infected with MADV include, but are not limited to, depression or hyperexcitability, somnolence, lethargy, circling, ataxia, head pressing, blindness, excessive chewing, decreased lingual tone, weakness, and paralysis, often accompanied by anorexia and progressive weight loss (5, 20, 21, 30, 31). None of these clinical signs were observed in the horses in this study. An interesting finding, however, was delayed proprioception in Horse 1 from 1 to 6 dpi and again at 10 dpi. Proprioceptive deficits are recognized indicators of neurological dysfunction in horses and may occur with diseases affecting the spinal cord or other areas of the central nervous system (32, 33). Although the deficits were mild and transient, their occurrence following MADV inoculation raises the possibility of limited neurological involvement. However, because no histopathological lesions were observed in the brain or spinal cord of either horse, this finding should be interpreted with caution. Whether the proprioceptive deficits reflected subtle neurological dysfunction without overt structural lesions (34) or were incidental remains unknown.

Several factors could explain the absence of overt clinical disease following MADV inoculation in the two horses. First, although moderate to high morbidity and case fatality rates have been reported during MADV epizootics (19), the morbidity rate of this particular MADV strain in horses is unknown, despite its isolation from a fatal equine case. If only a subset of infected horses develops clinically apparent disease, as occurs with many infectious agents, this outcome may not be observed in a study involving only two animals.

Second, passage of the virus in cell culture could have introduced genetic changes affecting its phenotype (29). Sequence analysis of the E2 region of the P11 stock identified an L29R substitution relative to the P5 sequence, likely acquired during cell-culture passage, which introduced a positively charged amino acid at residue 29. Mutations resulting in the acquisition of positively charged amino acids in E2 can arise during cell-culture passage and, in some alphaviruses, have been associated with increased heparan-sulfate binding and attenuation in vivo (29). However, positively charged E2 substitutions have also been associated with the emergence of equine-virulent VEEV strains (17, 18), while naturally occurring positively charged residues involved in heparan-sulfate binding in NA EEEV have been associated with increased neurovirulence (35). Given these contrasting effects, which may depend on the virus and the position of the substitution (29), the effect of L29R on MADV virulence cannot be inferred from our data. Furthermore, because only E2 was sequenced, additional mutations may have occurred elsewhere in the viral genome during cell-culture passage and could have affected viral phenotype.

Another possibility is that the virus dose used for inoculation may have influenced the clinical outcome. Previous experimental studies with NA EEEV demonstrated a dose-dependent effect on the course of infection in horses. Horses receiving a higher inoculum developed detectable viremia and, in some cases, severe or fatal neurological disease, whereas horses receiving the lowest inoculum developed neither detectable viremia or fever but mounted low-level neutralizing antibody responses, consistent with subclinical infection (10). The low inoculum used in our study was selected based on previous experimental studies involving alphavirus infection in horses, in which a similar dose (2,000 PFU) was used to approximate the amount of virus delivered by a single infected mosquito during feeding (14, 15). However, horses exposed during an epizootic may receive multiple infectious mosquito bites, potentially resulting in a substantially greater cumulative virus dose than that administered in our study.

Previous exposure to NA EEEV antigens could also have influenced the clinical outcome. Although Horse 1 was seronegative and Horse 2 had a very low NA EEEV-neutralizing antibody titer, not indicative of protective immunity against NA EEEV (36), given the horses' age, previous vaccination or natural exposure through mosquito bites cannot be excluded. Because protection following vaccination with inactivated NA EEEV vaccines can persist in the absence of detectable neutralizing antibodies (9), and cross-protection against MADV has been observed in mice vaccinated with an inactivated NA EEEV vaccine despite the absence of detectable MADV-neutralizing antibodies (28, under review), a contribution of pre-existing immunity to the clinical outcome, although unlikely, cannot be excluded.

Conclusions

This work demonstrated the feasibility of conducting experimental infection with a neurotropic alphavirus and direct mosquito-feeding procedures in horses under high-containment conditions. Although needle inoculation was used in this pilot study to ensure that both horses received the same virus dose, future studies could replicate the complete transmission cycle by infecting horses through the bites of infected mosquitoes and subsequently allowing uninfected mosquitoes to feed on the infected horses, as performed in previous experimental studies (10, 22). This approach would enable evaluation of both mosquito-to-horse and horse-to-mosquito transmission within the same experimental system. The model established here can also be applied to MADV and other equine alphaviruses to investigate differences in pathogenesis, determinants of transmission, alternative routes of transmission (including respiratory shedding), vaccine efficacy and duration of immunity, and novel preventive and therapeutic interventions. Such studies may be particularly valuable for improving our understanding and control of emerging and re-emerging arboviruses that affect both equids and humans.

Declarations

Ethics approval

All animal procedures were reviewed and approved by the Texas A&M University Institutional Animal Care and Use Committee (protocol IACUC 2024-0306). All procedures involving MADV were conducted

in accordance with institutional biosafety requirements and under protocols approved by the TAMU Institutional Biosafety Committee (protocol IBC2022-075 (RG3)).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This research was funded by Texas A&M AgriLife Research (PI: T.M.).

Authors' contributions

TM conceptualized the study, and LT participated in the experimental design. TM and LT co- supervised the experimental phase of the horse trials, from development of the experimental protocols and completion of the Institutional Animal Care and Use Committee (IACUC) and biosafety approval processes through conduct of the experiments, jointly performed the mosquito-feeding procedures on the horses, and analyzed the study data. LT served as the lead equine veterinarian throughout the study, performed the clinical evaluations, and collected blood and other samples from the horses. TM managed the mosquitoes and performed the molecular assays on horse plasma and mosquito pools. AC performed horse euthanasia and necropsies, tissue collection, tissue trimming, and histological examination. WSIV conducted the field collection of mosquitoes used in the study. LVS performed the E2 sequence analysis and contributed to interpretation of the sequencing results. WT performed the PRNT assays, with oversight from GH, and both contributed to interpretation of the PRNT results. TM and LT analyzed the study data and interpreted the overall findings. LT and TM wrote the first draft of the manuscript, and all authors contributed to manuscript review and editing.

Acknowledgements

We are grateful to the Texas A&M University Institutional Animal Care and Use Committee (IACUC) and Biosafety teams for their support in the development and optimization of the protocols used in this study, and to Dr. Kurt Zuelke, Dr. Melissa Kahl-McDonagh, Jessica Cobos, Nicola Benning, Sharkon Wright, Elissa Emmons, Bailey Jenson, Bryan Reagan, Stephen Prince, and other team members of the Global Health Research Complex team for their contributions to the development and conduct of this study. We also thank Drs. Robert Rose and Jonathan Bova at the Texas A&M University Comparative

Medicine Program for their contributions to protocol optimization and guidance on animal health and welfare, Dr. Raquel Rech for the histological analysis of the central nervous tissues of the two horses, and Dr. Erika Schwartz at Texas A&M Veterinary Medical Diagnostic Laboratory for assistance with diagnostic testing for equine encephalitic viruses.

References

1. Aguilar PV, Estrada-Franco JG, Navarro-Lopez R, Ferro C, Haddow AD, Weaver SC. Endemic Venezuelan equine encephalitis in the Americas: hidden under the dengue umbrella. Future Virol. 2011;6(6):721–40.
2. Arrigo NC, Adams AP, Weaver SC. Evolutionary patterns of eastern equine encephalitis virus in North versus South America suggest ecological differences and taxonomic revision. J Virol. 2010;84(2):1014–25.
3. Calisher CH, Karabatsos N, Lazuick JS, Monath TP, Wolff KL. Reevaluation of the western equine encephalitis antigenic complex of alphaviruses (family Togaviridae) as determined by neutralization tests. Am J Trop Med Hyg. 1988;38(2):447–52.
4. Calisher CH. It Isn't Going to Reach Here: Venezuelan Equine Encephalitis in Texas, 1971. In: Vasilakis N, Kramer LD, editors. History of Arbovirology: Memories from the Field. Online: Springer Nature; 2023.
5. Carrera JP, Forrester N, Wang E, Vittor AY, Haddow AD, Lopez-Verges S, et al. Eastern equine encephalitis in Latin America. N Engl J Med. 2013;369(8):732–44.
6. Vissani MA, Alamos F, Tordoya MS, Minatel L, Schammas JM, Dus Santos MJ et al. Outbreak of Western Equine Encephalitis Virus Infection Associated with Neurological Disease in Horses Following a Nearly 40-Year Intermission Period in Argentina. Viruses. 2024;16(10).
7. Steele KE, Twenhafel NA. REVIEW PAPER: pathology of animal models of alphavirus encephalitis. Vet Pathol. 2010;47(5):790–805.
8. Bowen GS, Fashinell TR, Dean PB, Gregg MB. Clinical aspects of human Venezuelan equine encephalitis in Texas. Bull Pan Am Health Organ. 1976;10(1):46–57.
9. Barber TL, Walton TE, Lewis KJ. Efficacy of trivalent inactivated encephalomyelitis virus vaccine in horses. Am J Vet Res. 1978;39(4):621–5.
10. Kissling RE, Chamberlain RW, Eidson ME, Sikes RK, Bucca MA. Studies on the North American arthropod-borne encephalitides. II. Eastern equine encephalitis in horses. Am J Hyg. 1954;60(3):237–50.
11. Tenbroeck C, Hurst EW, Traub E. Epidemiology of Equine Encephalomyelitis in the Eastern United States. J Exp Med. 1935;62(5):677–85.
12. Henderson BE, Chappell WA, Johnston JG Jr., Sudia WD. Experimental infection of horses with three strains of Venezuelan equine encephalomyelitis virus. I. Clinical and virological studies. Am J Epidemiol. 1971;93(3):194–205.

13. Sudia WD, Newhouse VF, Henderson BE. Experimental infection of horses with three strains of Venezuelan equine encephalomyelitis virus. II. Experimental vector studies. Am J Epidemiol. 1971;93(3):206–11.
14. Wang E, Bowen RA, Medina G, Powers AM, Kang W, Chandler LM, et al. Virulence and viremia characteristics of 1992 epizootic subtype IC Venezuelan equine encephalitis viruses and closely related enzootic subtype ID strains. Am J Trop Med Hyg. 2001;65(1):64–9.
15. Gonzalez-Salazar D, Estrada-Franco JG, Carrara AS, Aronson JF, Weaver SC. Equine amplification and virulence of subtype IE Venezuelan equine encephalitis viruses isolated during the 1993 and 1996 Mexican epizootics. Emerg Infect Dis. 2003;9(2):161–8.
16. Zehmer RB, Dean PB, Sudia WD, Calisher CH, Sather GE, Parker RL. Venezuelan equine encephalitis epidemic in Texas, 1971. Health Serv Rep. 1974;89(3):278–82.
17. Brault AC, Powers AM, Holmes EC, Woelk CH, Weaver SC. Positively charged amino acid substitutions in the e2 envelope glycoprotein are associated with the emergence of venezuelan equine encephalitis virus. J Virol. 2002;76(4):1718–30.
18. Anishchenko M, Bowen RA, Paessler S, Austgen L, Greene IP, Weaver SC. Venezuelan encephalitis emergence mediated by a phylogenetically predicted viral mutation. Proc Natl Acad Sci U S A. 2006;103(13):4994–9.
19. Magalhaes T, Hamer GL, de Carvalho-Leandro D, Ribeiro VML, Turell MJ. Uncertainties Surrounding Madariaga Virus, a Member of the Eastern Equine Encephalitis Virus Complex. Vector Borne Zoonotic Dis. 2024;24(10):633–40.
20. Campos KF, Oliveira CHS, Reis AB, Yamasaki EM, Brito MF, Andrade SJT, et al. Surto de encefalomielite equina Leste na Ilha de Marajó, Pará. Pesq Vet Bras. 2013;33(4):443–8.
21. Silva ML, Galiza GJ, Dantas AF, Oliveira RN, Iamamoto K, Achkar SM, et al. Outbreaks of eastern equine encephalitis in northeastern Brazil. J Vet Diagn Invest. 2011;23(3):570–5.
22. Bunning ML, Bowen RA, Cropp CB, Sullivan KG, Davis BS, Komar N, et al. Experimental infection of horses with West Nile virus. Emerg Infect Dis. 2002;8(4):380–6.
23. Gil L, Magalhaes T, Santos B, Oliveira LV, Oliveira-Filho EF, Cunha JLR et al. Active Circulation of Madariaga Virus, a Member of the Eastern Equine Encephalitis Virus Complex, in Northeast Brazil. Pathogens. 2021;10(8).
24. Carvalho-Leandro D, Ferreira FC, Fernandez Santos N, Sames WJt, Tian Y, Ndeffo-Mbah ML, et al. Transmission potential of Culex and Aedes species for Madariaga virus, a member of the eastern equine encephalitis virus complex. PLoS Negl Trop Dis. 2026;20(5):e0013516.
25. Causey OR, Shope RE, Sutmoller P, Laemmert H. Epizootic eastern equine encephalitis in the Bragança region of Pará, Brazil. Rev Serv Esp Saúde Pública. 1962;12(1):39–45.
26. Vasconcelos PFC, Travassos da Rosa JFS, Travassos da Rosa APA, Degallier N, Pinheiro FP, Sá Filho GC. Epidemiologia das encefalites por arbovírus na amazônia brasileira. Rev Inst Med Trop S Paulo. 1991;33(6):465–76.

27. Young A. Neurological Examination in the Horse Online: University of California, Davis; 2021 [Available from: https://cehhorsereport.vetmed.ucdavis.edu/news/neurological-examination-horse
28. Cerezo SA, Sung C, Tang WW, Hamer GL, Rech RR, Magalhaes T. Eastern equine encephalitis virus-vaccinated mice are protected against Madariaga virus despite negligible neutralizing antibody titers. In: University TAM, editor. Under review2026.
29. Alcorn MDH, Klimstra WB. Glycosaminoglycan binding by arboviruses: a cautionary tale. J Gen Virol. 2022;103(2).
30. Sousa SKH, Sonne L, Sant'Ana FJF, Reis Jr. JL. Encefalomielite equina do leste no Distrito Federal e entorno. Acta Sci Vet. 2015(43):1–7.
31. Medina G, Gleiser CA, Mackenzie RB. Outbreak of equine encephalomyelitis in the Republic of Panama. Bol Oficina Sanit Panam. 1965;58(5):390–4.
32. Gomez DE, Suarez G, de Tonnerre DJ, Mallicote MF, Hernadez JA, Sanchez LC et al. Eastern equine encephalitis-104 horses (1979–2017). J Vet Intern Med. 2026;40(3).
33. Hahn C. Neurological Examination of Horses. Vet Clin North Am Equine Pract. 2022;38(2):155–69.
34. Agner SC, Klein RS. Viruses have multiple paths to central nervous system pathology. Curr Opin Neurol. 2018;31(3):313–7.
35. Gardner CL, Ebel GD, Ryman KD, Klimstra WB. Heparan sulfate binding by natural eastern equine encephalitis viruses promotes neurovirulence. Proc Natl Acad Sci U S A. 2011;108(38):16026–31.
36. Desanti-Consoli H, Bouillon J, Chapuis RJJ. Equids' Core Vaccines Guidelines in North America: Considerations and Prospective. Vaccines (Basel). 2022;10(3).

Figures



Figure 1

Mosquito-feeding procedure in horses. Image from Trial 1 showing a 16-oz carton containing female mosquitoes secured to a horse's neck for mosquito feeding. Cartons containing 50-80 female mosquitoes were secured in this manner, and mosquitoes were allowed to feed for 30 min. The same procedure was used in Trial 2, during which one or two cartons were used depending on the day, with each carton containing one mosquito species (*Aedes taeniorhynchus* or *Aedes aegypti*).



Figure 2

Delayed proprioception observed in a horse following Madariaga virus (MADV) inoculation. Horse 1 exhibited delayed hindlimb proprioception on days 1–6 and 10 post-inoculation. The relationship between this finding and MADV infection cannot be determined from this study.



Figure 3

Local reaction to mosquito bites. One horse (Trials 1 and 2) exhibited a mild to moderate local reaction to mosquito bites on the neck. The image shows the reaction immediately after mosquito feeding, which resolved completely without intervention within 24 h.

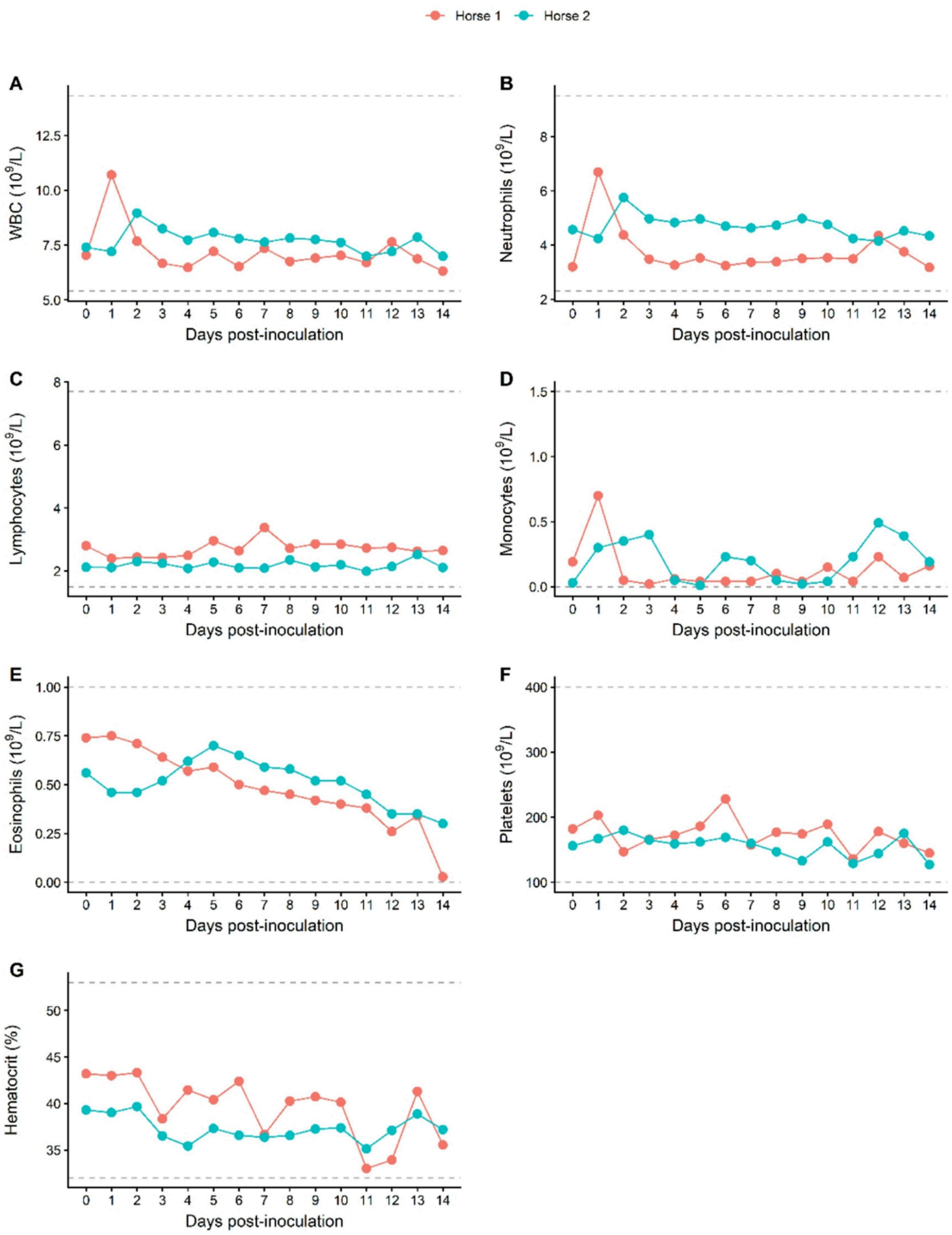


Figure 4

Hematological parameters following experimental challenge with Madariaga virus (MADV). A) Total white blood cell (WBC) counts; B) Absolute neutrophil counts; C) Absolute lymphocyte counts; D) Absolute monocyte counts; E) Absolute eosinophil counts, F) Platelet counts; G) Hematocrit. Parameters were measured from 0 to 14 days post-inoculation (dpi) in two horses experimentally challenged with

MADV (Trial 2). Blood collection at 0 dpi was performed before virus inoculation. Dashed horizontal lines indicate the reference intervals provided by the hematology analyzer.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- MADV165E2P11.txt