Rift Valley fever virus (RVFV) causes serious disease in ruminants and humans in Africa. In North America, there are susceptible ruminant hosts and competent mosquito vectors, yet there are no fully licensed animal vaccines for this arthropod-borne virus, should it be introduced. Studies in sheep and cattle have found the attenuated strain of RVFV, MP-12, to be both safe and efficacious based on early testing, and a 2-year conditional license for use in U.S. livestock has been issued. The purpose of this study was to further determine the vaccine’s potential to infect mosquitoes, the duration of humoral immunity to 24 months postvaccination, and the ability to prevent disease and viremia from a virulent challenge. Vaccination experiments conducted in sheep found no evidence of a potential for vector transmission to 4 North American mosquito species. Neutralizing antibodies were elicited, with titers of >1:40 still present at 24 months postvaccination. Vaccinates were protected from clinical signs and detectable viremia after challenge with virulent virus, while control sheep had fever and high-titered viremia extending for 5 days. Antibodies to three viral proteins (nucleocapsid N, the N-terminal half of glycoprotein GN, and the nonstructural protein from the short segment NSs) were also detected to 24 months using competitive enzyme-linked immunosorbent assays. This study demonstrates that the MP-12 vaccine given as a single dose in sheep generates protective immunity to a virulent challenge with antibody duration of at least 2 years, with no evidence of a risk for vector transmission.

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**Evaluation of the Efficacy, Potential for Vector Transmission, and Duration of Immunity of MP-12, an Attenuated Rift Valley Fever Virus Vaccine Candidate, in Sheep**

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Rift Valley fever virus (RVFV) is a vector-borne Phlebovirus (Bunyaviridae) that causes a noncontagious disease of wild and domestic ruminants and a zoonotic disease of humans. The virus has a tripartite RNA genome with large (L), medium (M), and small (S) segments (1). Rift Valley fever (RVF) is typically mild or unapparent in adult ruminants but causes abortion storms and high fatality rates in neonates. In humans, the disease may be undetected or exhibited by mild fever and flu-like symptoms; however, ocular disease, meningoencephalitis (2), and hemorrhagic fever syndromes are also possible, with case fatality rates ranging from 1% to 25% (3, 4). Transmission between ruminants is by mosquitoes, while in humans, infection is often acquired by direct contact with tissues and fluids from infected animals (5).

RVFV was first identified in 1930 in sub-Saharan Africa where it is endemic and causes periodic epizootics (6). It has shown the capability to expand into new regions by spreading into Egypt in 1977, causing an epidemic with high morbidity and mortality in livestock and humans, and to the Arabian Peninsula in 2000-2001 (7–9). Many mosquito species are competent vectors of the virus, including species present in North America (10–13), and stable flies and house flies are potential mechanical vectors (14). The range of competent vectors and susceptible ruminant hosts in the United States might contribute to the rapid spread and establishment of the virus if it were introduced, resulting in a significant threat to the livestock industry and public health (15, 16). RVFV has been classified as a category A high-priority pathogen by the National Institute for Allergy and Infectious Diseases and is dual listed as a U.S. Department of Agriculture (USDA) select agent due to the high risk to the U.S. livestock industry (17). A safe and effective vaccine for use in animals is needed to prevent disease and control the spread of infections in the case of an accidental or intentional introduction into North America (18); however, there are currently no fully approved RVFV human or animal vaccines for use in this continent.

An ideal vaccine for livestock should be safe, provide rapid long-lasting protection from infection with a single dose, and prevent viremia sufficient for transmission by competent vectors (19). Modified live virus (MLV) vaccines and killed virus vaccines are used widely in areas of Africa where RVFV is endemic (20, 21). Modified live virus vaccines are attractive for use in livestock since they generally elicit rapid onset of protective immunity with a single dose compared with killed or subunit vaccines that require additional injections.

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In challenge (29,30). MP-12 was avirulent when given to 7-day-old lambs born from these ewes became antibody positive after ingestion of colostrum and were passively protected from a virulent virus and resulted in production of neutralizing antibodies. Moreover, 1 USAMRIID source vaccine (31) and 2-week-old lambs (32) and protected them from disease after vaccination. Some of these experiments found that the vaccine is safe and effective in cattle (34,35). A single challenge with virulent vector or animal host. Vector competence for transmission of vector-borne viruses with the potential for reassortment of other viruses.

An attenuated RVFV vaccine candidate, MP-12, was previously generated by 12 serial passages of the wild-type ZH548 strain of RVFV in the presence of a chemical mutagen (22) and selected based on attenuation of virulence in mice and hamsters (23). Importantly, mutations were identified in all three genomic segments and attenuating features were found on segments M and L (24–26), thus significantly reducing the risk of reversion to virulence that might otherwise arise from a recombination event with other viruses.

Previous work has been done to test MP-12 for use as an animal vaccine, and recent studies found that the vaccine is safe and protective from a virulent challenge in nonhuman primates (27, 28). In sheep, immunization of pregnant ewes did not cause abortion and resulted in production of neutralizing antibodies. Moreover, lambs born from these ewes became antibody positive after ingestion of colostrum and were passively protected from a virulent challenge (29, 30). MP-12 was avirulent when given to 7-day-old (31) and 2-week-old lambs (32) and protected them from disease after a virulent challenge 14 days postvaccination. Some of these lambs had transient low-titer MP-12 viremia (<3 log10 PFU/ml) postvaccination, but an artificial vector feeding trial indicated that this titer was unlikely to infect the insect vector (33). Studies also found MP-12 to be safe and effective in cattle (34, 35). A single study found abortions in sheep vaccinated during the first trimester of pregnancy; however, these sheep were housed outside under uncontrolled conditions in an area where RVFV is endemic, so there may have been other contributing factors (36). The USDA Center for Biologics has issued a 2-year conditional license for MP-12 based on the reasonable expectation of safety and efficacy (37). A potential next-generation vaccine, an RVF MP-12 gene-deleted vaccine, was found to be safe in pregnant sheep (38).

A component of safety testing of a modified live virus vaccine is multiple “booster” injections. A concern with an MLV vaccine is the potential for reversion to virulence, especially in the instance of vector-borne viruses with the potential for reassortment of multisegmented genomes with related wild-type virus in a coinfected vector or animal host. An attenuated RVFV vaccine candidate, MP-12, was previously generated by 12 serial passages of the wild-type ZH548 strain of RVFV in the presence of a chemical mutagen (22) and selected based on attenuation of virulence in mice and hamsters (23). Importantly, mutations were identified in all three genomic segments and attenuating features were found on segments M and L (24–26), thus significantly reducing the risk of reversion to virulence that might otherwise arise from a recombination event with other viruses.

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A component of safety testing of a modified live virus vaccine is to determine the potential for vector transmission of the vaccine to another vertebrate host. Vector competence for transmission requires multiple sequential steps; infection of the insect must be established and disseminate beyond the midgut, and the virus must overcome the salivary gland barrier to allow for transmission. The completion of this process is the extrinsic incubation period, which has been shown to range from 8 to 20 days for RVFV virulent and attenuated strains (11, 13). Early studies found that the low titer (<10^3 PFU/ml) of MP-12 detected in some animals postvaccination is probably insufficient to establish an infection in mosquitoes (33).

The aim of this study was to extend the safety and efficacy testing of the RVFV vaccine, MP-12, in order to support the full licensure for use in the United States. This included determining the long-term duration of neutralizing antibodies in sheep and protection from a virulent challenge and determination of the potential for vaccine transmission from vaccinated sheep to four mosquito species common in the United States.

**MATERIALS AND METHODS**

**Animals.** Institutional animal care and use committees from the Canadian Food Inspection Agency or the University of Wyoming approved all animal studies. Forty conventional mixed-breed sheep were vaccinated with RVFV MP-12 in four separate studies, while 32 randomly selected cohorts served as nonvaccinated controls (Table 1). All sheep were purchased from reputable breeders and housed under insect-proof biological safety level (BSL)-2 (experiments 1 to 3) or BSL-3 Ag (experiment 4) conditions. Four mice were immunized with MP-12 to generate polyclonal antibodies for use in competitive enzyme-linked immunosorbent assays (cELISAs).

**Virus and vaccine strains.** The RVFV MP-12 strains were provided by the U.S. Army Medical Research Institute for Infectious Diseases (USAMRIID), designated uMP-12 (vaccine used in previously published studies), and, when available, by the vaccine manufacturer (Zoetis, Florham Park, NJ), designated zMP-12. The manufacturer-prepared MP-12 only became available for experiments 3 and 4. uMP-12 was propagated 1 time in fetal lung fibroblast MRC-5 cell cultures (American Tissue Culture Collection [ATCC], Manassas, VA) in medium 199 with Earle’s salts

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**TABLE 1 Summary of four MP-12 vaccine trials in sheep**

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Vaccine source (dose, PFU)</th>
<th>Expt description</th>
<th>Sheep</th>
<th>Samples</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>USAMRIID source vaccine (2.9 × 10^6)</td>
<td>Vaccine virus detection in tissue samples at 3–4 days postvaccination and vector transmission</td>
<td>4 vaccinates, 2 controls</td>
<td>Blood, liver, and spleen; mosquitoes fed on sheep</td>
<td>RT-PCR, virus isolation</td>
</tr>
<tr>
<td>2</td>
<td>USAMRIID source vaccine (2.9 × 10^6)</td>
<td>Antibody response and vector transmission study</td>
<td>10 vaccinates, 8 controls</td>
<td>Blood samples; mosquitoes fed on sheep</td>
<td>PRNT_70, virus isolation, RT-PCR</td>
</tr>
<tr>
<td>3</td>
<td>Manufacturer source vaccine (1.35 × 10^6)</td>
<td>Long-term antibody detection and vector transmission</td>
<td>10 vaccinates, 10 controls</td>
<td>Blood samples; mosquitoes fed on sheep</td>
<td>PRNT_70, cELISA, RT-PCR, virus isolation</td>
</tr>
<tr>
<td>4</td>
<td>Manufacturer source freeze-dried vaccine (10^7)</td>
<td>Challenge with virulent virus</td>
<td>16 vaccinates, 4 challenge controls</td>
<td>Blood and tissue samples postchallenge with ZH501</td>
<td>PRNT_70, virus isolation, RT-PCR</td>
</tr>
</tbody>
</table>

a Sheep were vaccinated with the Rift Valley fever virus modified live virus vaccine, MP-12, in four trials.
TABLE 2 Results of vector infection studies

<table>
<thead>
<tr>
<th>Expt no. or control</th>
<th>Mosquito species (no.)</th>
<th>No. of sheep</th>
<th>Assay</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Aedes aegypti</em> (35)</td>
<td>4</td>
<td>RT-PCR</td>
<td>Body and legs negative</td>
</tr>
<tr>
<td></td>
<td><em>Culex quinquefasciatus</em> (15)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>Aedes taeniorhynchus</em> (566)</td>
<td>5</td>
<td>RT-PCR</td>
<td>Body and legs negative</td>
</tr>
<tr>
<td></td>
<td><em>Culex tarsalis</em> (149)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>Aedes taeniorhynchus</em> (231)</td>
<td>5</td>
<td>RT-PCR</td>
<td>Body and legs negative</td>
</tr>
<tr>
<td></td>
<td><em>Culex tarsalis</em> (172)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td>Mosquitoes of each species (n = 50) fed on nonvaccinated sheep</td>
<td>12</td>
<td>VI</td>
<td>Body and legs negative</td>
</tr>
<tr>
<td>Positive control</td>
<td>Mosquitoes (n = 47) fed MP-12-spiked blood (10^9–10^10 PFU/ml), tested on day of feeding</td>
<td>NA(^b)</td>
<td>VI</td>
<td>Positive</td>
</tr>
</tbody>
</table>

\(^a\) Four species of mosquitoes common to North America were allowed to feed on MP-12-vaccinated sheep on days 2 to 4 after vaccination and held for 10 to 14 days prior to individual testing for vaccine virus by RT-PCR. Bodies and legs of the mosquitoes were separated and tested separately. Some mosquitoes were artificially fed a blood meal spiked with up to 10^6 PFU/ml MP-12 and held for 14 days prior to testing by virus isolation (VI). Positive-control mosquitoes were sampled whole on the day of the spiked blood meal.

\(^b\) NA, not applicable.

(M199-E [Sigma-Aldrich, St. Louis, MO]; 100 U/ml penicillin and 100 µg/ml streptomycin sulfate [Sigma-Aldrich]) with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA) and was titrated using a plaque assay as described previously (39). The manufacturer-supplied MP-12 was received as a master seed stock cell culture material (7.4 log 30% tissue culture infective dose [TCID\(_{50}\)/ml] for experiment 3) and as a lyophilized vaccine for experiment 4. The wild-type RVFV strain ZH501 (40), originally provided by Heinz Feldmann, National Microbiology Laboratory, Winnipeg, Canada, was propagated in *Aedes albopictus* C6/36 cells (ATCC) for use in animal infections and titrated in Vero E6 cells (ATCC) as previously described (41). The C6/36 cells were maintained in 47% ESF-921 (Expression Systems, Davis, CA)-47% Eagle’s minimal essential medium (EMEM)-2.5% FBS (Wisent, St-Bruno, QC, Canada)-2.5% HEPES (25 mM final)-1% sodium pyruvate (1 mM final) (Sigma-Aldrich) at 28°C in phenolic style cap or plug seal cap flasks (Corning, Corning, NY).

Experimental design. Four experiments were performed using sheep vaccinated with RVFV MP-12, and mock-vaccinated cohorts served as controls. Experiments 1 and 2 used the MP-12 vaccine strain used in previously published studies (uMP-12). The manufacturer-prepared vaccine (zMP-12) became available and was used for experiments 3 and 4. Sheep numbers and treatments are listed in Table 1. All vaccinations were administered subcutaneously at the shoulder.

(i) Experiment 1. A short-term experiment was conducted to test for the potential to detect vaccine virus in tissues postvaccination and for the potential to infect mosquitoes by feeding on vaccinated sheep. Four ewes were vaccinated with 1 ml of uMP-12 containing 2.9 × 10^9 PFU/ml diluted in phosphate-buffered saline (PBS) immediately prior to use, and 2 control ewes received diluent only. Wool was shaved from the axillary region to allow for mosquito feeding. *Aedes aegypti* and *Culex quinquefasciatus* in mesh-top containers were allowed to feed on all sheep for 20 min on 2, 3, and 4 days postvaccination (dpv), held for 10 to 14 days, and tested for RVFV by reverse transcription (RT)-PCR. Blood samples were collected from sheep prior to vaccination and daily until necropsy at 3 or 4 dpv, when liver, spleen, and blood samples were collected and frozen at −20°C until testing by RT-PCR and virus isolation within 2 weeks of sampling.

(ii) Experiment 2. Ten vaccinated and 8 control 2-month-old lambs were treated as in experiment 1. *Aedes taeniorhynchus* and *Culex tarsalis* were allowed to feed on 5 each of the vaccinated and control sheep 2 to 4 dpv as described for experiment 1. Blood samples were collected on 1 to 7, 14, 21, and 28 dpv and tested for uMP-12 by virus isolation and RT-PCR. Serum was tested for neutralizing antibodies by a plaque reduction assay. Sheep were observed daily for normal activity and appetite, and rectal temperatures were taken prior to vaccination and at 1 to 5 dpv.

(iii) Experiment 3. Ten 4-month-old lambs were vaccinated using zMP-12 master seed stock with titer determined in PFU as 1.35 × 10^9 PFU/ml, diluted according to company recommendations at 1:100 in Dulbecco’s modified Eagle medium (DMEM) (Gibco, Carlsbad, CA) immediately prior to use and given as a 1-ml dose. Ten negative-control lambs received diluent only. Four vaccinates received a second dose 40 days after the primary vaccination. *Aedes taeniorhynchus* and *Culex tarsalis* were allowed to feed on 5 each of the vaccinated and control sheep on 2 to 4 dpv as described above. Activity and appetite were monitored, rectal temperatures were taken daily prior to vaccination and at 1 to 7 dpv, and blood samples were collected on days 0, 2, 3, 4, 7, and 10 and weekly to 60 dpv. The sheep were then moved to outdoor pens and maintained to 24 months postvaccination with blood samples collected every 60 days for testing for neutralizing antibodies by a plaque reduction assay.

(iv) Experiment 4. Sixteen 3-month-old lambs received lyophilized zMP-12 vaccine diluted to 3 × 10^6 PFU/2.5 ml with stabilizer (20% lactose, 20% gelatin, 16% N-Z-Amine, extender [HALS], 45 mg/ml gentamicin). The dosage and preparation were based on the directions provided by the manufacturer. Four cohorts received diluent only and served as negative controls housed with the vaccinates. All lambs were challenged with virulent virus 21 dpv. The rectal temperature was monitored daily, prior to vaccination, postvaccination, and postchallenge. Blood samples from all sheep were collected prior to vaccination and at 1, 7, 14 and 21 dpv to monitor neutralizing antibodies in serum by a plaque reduction assay. All animals were challenged at 21 dpv with 1 ml of RVFV ZH501 (2 × 10^7 PFU/ml) with the dosage verified by back titration on Vero E6 cells. Blood samples were collected prior to challenge and then daily until 6 days postinfection (dpi) when animals were euthanized, and liver and spleen were collected for virus isolation and virus RNA detection by RT-PCR.

Mosquitoes. Early studies with MP-12 found transient low-titered viremia (<10^3 PFU/ml) in some vaccinates, a level thought to be insufficient to establish an infection in mosquitoes (31, 33). We further tested this assumption by allowing mosquitoes to feed on vaccinates or controls on days 2 to 4 postvaccination. Mosquitoes then were held for 10 to 14 days to allow for the clearance of the blood meal and amplification of the virus. Mosquito legs and bodies were tested separately by plaque assays on Vero cells to determine the extent of an infection. If an infection was established but failed to cross the midgut barriers, only the body would test positive, but legs and body would both be positive in a disseminated infection (13).

The mosquito species and numbers used in the study are listed in Table 2. The sources of the mosquitoes were as follows: *Aedes aegypti*, the Liverpool strain from a colony temporarily maintained at the Arthropod-Borne Animal Diseases Research Unit (ABADRU), Manhattan, KS; *Culex
**quinceaeasiatas**, from the Center for Medical, Agricultural and Veterinary Entomology (CMAVE), Gainesville, FL; *Culex tarsalis*, from an ABADRU colony originally established in California; and *Aedes taeniorhynchus*, obtained as eggs from CMAVE. Mosquitoes were raised to adults and maintained at 24 ± 2°C on a 10% sucrose solution with a 12-h photoperiod. Prior to feeding on sheep, mosquitoes were anesthetized using CO₂ for 10 to 15 s and placed in Plexiglas containers topped with fine-mesh fabric screens that allowed them to feed on the exposed skin of vaccinated sheep. Postfeeding, blood-fed females were sorted and maintained for 10 to 14 days, prior to individual testing for MP-12 by RT-PCR. Positive and negative controls included mosquito pools spiked with MP-12 and mosquitoes fed on the nonvaccinated sheep.

To determine the dose of zMP-12 in blood that was infectious to mosquitoes, *Culex tarsalis* and *Aedes taeniorhynchus* were fed defibrinated sheep blood spiked with 3, 4, and 5 log₁₀ PFU/ml of zMP-12 using the Hemotek feeding system (Discovery Workshops, Accrington, United Kingdom). Five to 10 blood-fed mosquitoes of each species at each zMP-12 dose were frozen at −80°C on the day of feeding as positive controls. The remaining blood-fed mosquitoes (*n = 86*) were sorted and maintained for 14 days and then tested individually for the presence of virus by a plaque assay.

**Antibody detection assays.** Serum neutralization antibodies were measured using 70% plaque reduction titer (PRNT₇₀) tests as previously described (31). Briefly, sera were heat treated for 30 min at 56°C and diluted 1:10, and then serial 4-fold dilutions were made in complete medium. Diluted sera were then incubated with an equal volume of medium with 100 PFU of virus for 60 min at 37°C prior to inoculation onto monolayers of Vero cells. After 1 h of incubation at 37°C for adherence, cells were overlaid with complete medium containing 0.5% SeaKem ME agarose (Sigma-Aldrich) and incubated for 3 days prior to overlay with complete medium with agarose containing 0.1 mg/ml neutral red dye and incubation for an additional 2 days. Plaques were counted, and titers were calculated as the reciprocal of the endpoint dilution that reduced the plaque count by ≥70% of the negative control. Experiments 1 to 3 were performed in BSL-2 against the attenuated uMP-12 strain, and experiment 4 was performed in BSL-3 against wild-type ZH501. Neutralization titers obtained against the virulent virus ZH501 and attenuated MP-12 were compared using serum samples from each experiment collected at 21 dpv and 6 dpi.

For experiment 3, antibodies were also tested using cELISAs developed as potential diagnostic tools to detect antibodies to three *in vitro* expressed RVFV proteins: N-terminal half of glycoprotein GN (GNn), nucleocapsid protein (N), and nonstructural protein from the S segment (NSs). Four mice were immunized twice at a 4-week interval by intraperitoneal injections of 0.1 ml containing 2.4 × 10⁵ log₉ UMP-12 in PBS. Blood samples were collected 4 weeks following the final booster, and polyclonal serum was separated and stored at −80°C until use in the cELISAs. Proteins used in the assay were produced by *in vitro* methods. The genetic sequence for GNn was acquired from the RVFV medium segment cloned into pWGR7077 plasmid, obtained from C. Schmaljohn (USAMRIID). The N and NSs protein coding sequences were acquired from the RVFV small gene segment cloned into pcDNA 3.1 and pQE-9, respectively, both obtained from F. Weber, Marburg University, Germany. Protein coding sequences were subcloned into the pET-30 Ek/LIC expression vector (Novagen, Darmstadt, Germany), and proteins were produced in BL21 cells. GNs and N proteins were purified by a nickel affinity column; the N-terminal His tag was cleaved using the enterokinase cleavage capture kit (Novagen) according to the manufacturer’s directions, and NSs was purified by His-Trap chromatography with the Pharmacia fast protein liquid chromatography (FPLC) system (model AKTA P-920). The purified proteins were applied to the wells of 96-well plates at 0.5 to 1.0 pg/μl. Sheep serum samples from experiment 3 were diluted 1:10 and added to duplicate wells, incubated for 1 h at room temperature, and washed 3 times in wash buffer (PBS, 0.05% Tween 20, 0.1% bovine serum albumin) prior to the addition of 1:1,000 mouse anti-MP-12. After incubation at room temperature for 1 h, the wells were similarly washed and then incubated with 1:200 biotinylated goat anti-mouse horseradish peroxidase (HRP) and 1:200 streptavidin conjugates (Biogenex, Fremont, CA). Optical density (OD) measurement at 492 nm, following addition of α-phenylenediamine substrate, allowed calculation of the serum antibody percent inhibition (PI) titers. These were calculated as 1 − (test serum OD/negative serum OD) × 100.

**Virus isolation and titrations.** Virus isolation and titrations were performed using a plaque assay as previously described (38) to test for postvaccinal MP-12 virus in the sheep and mosquitoes (experiments 1 to 4) and for postchallenge wild-type ZH501 (experiment 4). Briefly, 400 μl/well of 10-fold serially diluted sample (virus stock, serum, or 10% [wt/vol] clarified tissue homogenates) in DMEM (Wisent) was applied to triplicate wells of confluent vero E6 cells in 12-well plates. Plates were incubated at 37°C in 5% CO₂ for 1 h and then inoculum was replaced with complete medium with a 2% carboxymethyl cellulose overlay (Sigma-Aldrich). Plates were formalin (10% Sigma-Aldrich) fixed and stained with crystal violet (0.5%, 80% ethanol, 20% PBS) after 4 days of incubation. Titers are reported as log₁₀ PFU.

**RNA extraction and RT-PCR.** For experiments 1 to 3, RNA was isolated from serum or 10% (wt/vol) tissue homogenates using the MagMAX-96 viral RNA isolation kit (Applied Biosystems, Foster City, CA), following the manufacturer’s recommendations, with final elution in 50 μl of RNase-free water. Extracted RNA was used in an RT-PCR assay utilizing primers and probes targeting the L gene as described previously (42) with the minor modification of changing the forward primer’s 14th nucleotide from G to A so it would exactly match the MP-12 sequence (43). For experiment 4, RNA was similarly isolated using TriPure reagent (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s instructions. Real time RT-PCR using the same protocol (42) was modified to a one-step procedure (39). Armored enterovirus RNA was used as an exogenous internal control, and a plasmid standard curve was set up to estimate the number of detected copies of viral RNA (39).

**Statistics.** Serum neutralization titers were calculated as geometric means by group for each time point. The Student’s t test (Excel 97, 2003) for independent samples was used for statistical comparisons between the groups, and differences with P values of <0.05 were considered significant. Comparisons of postchallenge RT-PCR copy numbers and infectious virus titers between vaccinates and controls in experiment 4 were tested using the Mann-Whitney U test (Social Science Statistics on-line calculator; [http://www.soscistatistics.com/tests/mannwhitney/Default.aspx](http://www.soscistatistics.com/tests/mannwhitney/Default.aspx)) for independent samples with nonnormal distribution.

**RESULTS**

**Vaccine safety and vaccine viremia.** Sheep vaccinated with MP-12 were monitored for adverse reactions postvaccination. Vaccinates maintained normal activity and appetite postvaccination, with no significant differences in behavior or daily temperature compared to those of control animals. Virus isolation and RT-PCR were used to detect postvaccinal MP-12 virus or virus RNA in tissues and serum. Vaccine virus was isolated at <3 log₁₀ PFU/g virus from a liver sample of one vaccinated sheep from experiment 1 at 3 dpv but was not isolated from the serum of any vaccinates. Only MP-12 RNA at <3 log₁₀ copy number was detected from the serum of two sheep at 1 dpv. The vaccine did not cause adverse reactions, and viremia was not detected postvaccination.

**Potential for vector transmission.** Mosquitoes were fed on vaccinated and control sheep 2 to 4 dpv to test the potential for vector infection with vaccine virus. All mosquitoes (*n = 1,168*) fed on sheep 2 to 4 dpv with MP-12 were negative by RT-PCR (Table 2). To determine the level of vaccine virus infectious to mosquitoes, *Culex tarsalis* (*n = 10*) and *Aedes taeniorhynchus* (*n = 76*) were fed on blood spiked with up to 5 log₁₀ PFU/ml of zMP-12, a...
dose 100 times the highest detected level in our study and in previous studies (29, 31). To confirm that virus was consumed with this meal, additional mosquitoes were sampled on the day of feeding and tested for infectious virus by a plaque assay, with 67% (n = 15) of mosquitoes fed the highest dose testing positive. However, the legs and bodies of mosquitoes held 14 days postfeeding were negative, showing that the virus failed to establish an infection at this dose.

Antibody response and duration postvaccination. Vaccinated from all experiments developed neutralizing antibodies with peak titers 14 to 21 dpv, and no antibodies were detected prior to vaccination or in control animals. In experiments 2 and 3, the maximum antibody response was reached by 21 dpv with geometric mean PRNT$_{70}$ values of 485 and 278 and standard errors of the mean (SEM) of 217 and 84, respectively. In experiment 3, vaccinated sheep were maintained for 2 years to follow antibody responses to MP-12-vaccinated sheep from experiment 3 to three RVFV expressed proteins: the nucleocapsid protein (NP), a truncated version of a surface glycoprotein GN (GNn), and the nonstructural protein from the small genome segment (NSs). Antibodies were measured to 24 months postvaccination and are expressed as percent inhibition of the test serum compared to the prevaccination control serum. The titer is based on percent inhibition of the negative control, calculated as $1 - (\text{test serum OD/negative serum OD}) \times 100$.

Protection from virulent challenge. None of the MP-12-vaccinated sheep developed clinical signs of disease after challenge with virulent virus, while challenge control animals experienced increases in rectal temperatures, in general between 1 and 4 dpi. Challenged vaccinated animals had increased rectal temperatures only at 1 dpi (Fig. 3). In sheep, an increase in rectal temperature can be used as one of the parameters to evaluate vaccine efficacy (41).

The 4 challenge control sheep in our study had viremia postchallenge that was not significantly different from that of 8 sheep from a previous study inoculated with the same challenge virus/dose/route (41), so data from all controls (n = 12) were compared to those of the 16 vaccinated challenged animals (Table 1, experiment 4). Infectious virus was isolated from the serum samples of all control sheep for up to 4 days postchallenge, and viral RNA was detected in the serum samples of all controls at 2 dpi (Fig. 4A and B). No infectious virus was isolated from the serum samples of vaccinated sheep postchallenge, and low levels of RNA were only detected at 1 dpi (Fig. 4A). In tissues, viral RNA of ZH501 was detected 6 dpi in liver and spleen samples from control sheep with 3.1 to 5.3 log$_{10}$ copy number/0.1 g of tissue, but only from spleen samples of

FIG 2 Measurement of antibodies to Rift Valley fever virus (RVFV) recombinant proteins N, GNn, and NSs using cELISAs shown as the geometric mean titers at months postvaccination. cELISAs were used to measure the antibody responses in MP-12-vaccinated sheep from experiment 3.

FIG 1 Geometric mean 70% plaque reduction neutralization titer (PRNT$_{70}$) of zMP-12-vaccinated sheep receiving one or two vaccinations. Ten sheep vaccinated in experiment 3 with the attenuated Rift Valley fever virus vaccine, zMP-12, were tested for neutralizing antibodies to MP-12 using PRNT$_{70}$ assays. Four sheep received a second vaccination 40 days after the primary vaccination, resulting in a temporary increase in antibody titer (amnestic response). Neutralization titers were measured to 24 months postvaccination to determine the rate and level of antibody decay. The inset figure illustrates the response. Neutralization titers were measured to 24 months postvaccination to confirm that virus was consumed with this meal, additional mosquitoes were sampled on the day of feeding and tested for infectious virus by a plaque assay, with 67% (n = 15) of mosquitoes fed the highest dose testing positive. However, the legs and bodies of mosquitoes held 14 days postfeeding were negative, showing that the virus failed to establish an infection at this dose.

Antibody response and duration postvaccination. Vaccinated from all experiments developed neutralizing antibodies with peak titers 14 to 21 dpv, and no antibodies were detected prior to vaccination or in control animals. In experiments 2 and 3, the maximum antibody response was reached by 21 dpv with geometric mean PRNT$_{70}$ values of 485 and 278 and standard errors of the mean (SEM) of 217 and 84, respectively. In experiment 3, vaccinated sheep were maintained for 2 years to follow the duration of the neutralizing antibodies. One of the vaccinated sheep died of unrelated causes at 11 months postvaccination. Twenty-four months postvaccination, the mean neutralizing titer of the 9 remaining vaccinated sheep was 186 (SEM, 74), and all titers were $>1:40$ (Fig. 1). Four sheep from experiment 3 received a second vaccination to compare a prime-boost dosage to a single dose. These sheep developed a transient 7-fold increase in the antibody titer (mean, 1,810; SEM, 480) 2 weeks after the booster dose. This increase in the antibody titer decreased to the level of the once-vaccinated sheep by 12 months postvaccination (Fig. 1). In experiment 4 (virulent challenge), vaccinated animals developed low levels of neutralizing antibodies at 14 dpv, most animals retained the same titer at 21 dpv (mean, 25), and the titer increased postchallenge to a mean of 60. Values for PRNT$_{70}$ titers compared between assays using MP-12 and ZH501 were 3,420 and 345, respectively. Three cELISAs detecting antibodies to RVFV-expressed proteins were used for serum samples from experiment 3. Anti-N and anti-GNn antibodies were detected as early as 7 dpv and had reached maximum levels by 10 dpv. The cELISA for the NSs protein was used on samples from 10 to 24 months postvaccination and had similar titers at these time points. Titers based on cELISA detection of antibodies to individual expressed proteins began to decline at 16 months postvaccination (Fig. 2). This varied from the antibody titers determined by plaque assays that were uniform from 12 to 24 months, probably due to multiple antigenic sites targeted by the neutralizing assay.
one vaccinated, challenged sheep with 3.1 log_{10} copy number. No virus was isolated from serum or tissues from vaccinated animals at any time postvaccination or postchallenge.

**DISCUSSION**

A vaccine effective against Rift Valley fever virus is a public health and agriculture priority. A live attenuated virus vaccine is best suited for use in livestock since it can provide long-term protection with a single dose, but the potential for vector transmission of the vaccine can be of concern.

In the present study, we further tested MP-12 for the potential to infect mosquitoes fed on sheep postvaccination. The attenuated RVFV MP-12 was only transiently detected by RT-PCR in some sheep postvaccination and was not detected in mosquitoes fed on vaccinates. The infectivity of MP-12 for mosquitoes was tested by artificial feeding of a spiked blood meal with up to 10^6 PFU/ml. MP-12 was only detected in mosquitoes immediately after this feeding and was not detected in these insects tested after a 10-day hold to allow for clearance of the blood meal and potential virus amplification, showing that this dose was insufficient to establish an infection. Previous insect transmission studies with virulent and avirulent RVFV strains also found that a very high dose was required to establish an infection, in most cases >10^9 PFU/ml. In our study, we tested to a level 100 times that of any previously reported postvaccinal MP-12 viremia (29, 31). Together these findings demonstrate that there is insufficient viremia of MP-12 postvaccination to infect the tested vectors.

Our study demonstrates that sheep vaccinated with a single dose of MP-12 develop neutralizing antibodies that are still present at 2 years postvaccination with neutralizing titers of ≥40, levels that have been shown to be protective (34, 44). After a virulent challenge, vaccinated sheep are protected from clinical disease and viremia. Prevention of viremia sufficient to infect the vector is critical for preventing further transmission of the wild-type virus. Previous studies in cattle, sheep, and nonhuman primates also found that MP-12 elicits neutralizing antibodies and protects from a virulent challenge (27–36). Our study demonstrates that in sheep, antibodies are still present at levels likely to be protective 2 years after a single dose of the vaccine.
An introduction of RVFV into North America might have enormous public health and economic impacts, and a safe and rapidly effective vaccine for use in livestock is needed. Although next-generation, genetically engineered vaccines are in development, safety and efficacy studies in their target species are limited, and licensing of these products has proven to be difficult. Our results, along with prior studies in the target hosts (ruminants and in humans), demonstrate that MP-12 or MP-12-derived vaccines are excellent candidate RVFV vaccines with the potential for immediate, safe, and effective use.

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