Rift Valley Fever Virus Vaccine Lacking the NSs and NSm Genes Is Safe, Nonteratogenic, and Confers Protection from Viremia, Pyrexia, and Abortion following Challenge in Adult and Pregnant Sheep

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Rift Valley fever virus (RVFV) is a mosquito-borne human and veterinary pathogen causing large outbreaks of severe disease throughout Africa and the Arabian Peninsula. Safe and effective vaccines are critically needed, especially those that can be used in a targeted one-health approach to prevent both livestock and human disease. We report here on the safety, immunogenicity, and efficacy of the ΔNSs-ΔNSm recombinant RVFV (rRVFV) vaccine (which lacks the NSs and NSm virulence factors) in a total of 41 sheep, including 29 timed-pregnant ewes. This vaccine was proven safe and immunogenic for adult animals at doses ranging from 1.0 × 10^3 to 1.0 × 10^5 PFU administered subcutaneously (s.c.). Pregnant animals were vaccinated with 1.0 × 10^4 PFU s.c. at day 42 of gestation, when fetal sensitivity to RVFV vaccine-induced teratogenesis is highest. No febrile reactions, clinical illness, or pregnancy loss was observed following vaccination. Vaccination resulted in a rapid increase in anti-RVFV IgM (day 4) and IgG (day 7) titers. No seroconversion occurred in cohoused control animals. A subset of 20 ewes progressed to full-term delivery after vaccination. All lambs were born without musculoskeletal, neurological, or histological birth defects. Vaccine efficacy was assessed in 9 pregnant animals challenged at day 122 of gestation with virulent RVFV (1.0 × 10^6 PFU intravenously). Following challenge, 100% (9/9) of the animals were protected, progressed to full term, and delivered healthy lambs. As expected, all 3 sham-vaccinated controls experienced viremia, fetal death, and abortion postchallenge. These results demonstrate that the ΔNSs-ΔNSm rRVFV vaccine is safe and nonteratogenic and confers high-level protection in sheep.

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postvaccination (p.v.) abortions or teratogenesis in livestock (10, 20). These drawbacks have precluded the widespread use of these vaccines among livestock producers, except in emergency situations in the face of a nascent outbreak. The explosive nature of RVFV outbreaks in areas of endemicity and the severe consequences of its accidental or intentional introduction into RVFV-free areas necessitate the development of novel vaccines and comprehensive strategies for use in both livestock and humans.

In order to overcome some of the inherent limitations of previous live-attenuated vaccines, we used reverse genetics to develop a recombinant RVFV, ΔNSs-ΔNSm rRVFV, which contains complete gene deletions of the 2 known RVFV virulence factors, the NSs and NSm genes (4, 5, 33, 35). The RVFV genome contains 3 negative-sense, single-stranded RNA segments: the S segment encoding the nucleocapsid protein and NSs, the M segment encoding the NSm and Gn-Gc glycoproteins, and the L segment containing the virus polymerase. The NSs protein mediates multiple functions in infected cells, including interferon (IFN) antagonism (3, 23), protein kinase R (PKR) degradation (18, 21), and interactions with host cell chromosome structures (25), thus playing a critical role in mammalian host pathogenesis by indirectly disrupting multiple aspects of the host cell antiviral response (11, 27, 33). The NSm gene is less well characterized, but while it is dispensable for efficient RVFV growth in both IFN-competent and -deficient cells (17, 34), it has been shown to be important for in vivo RVFV virulence in rodents (5). A putative role for NSm as an antagonist of the host cell apoptotic pathway has been reported (35).

The ΔNSs-ΔNSm rRVFV vaccine utilized here was generated directly from precisely defined cDNA plasmids, grows to high titers in cell culture, and is of exact molecular identity and high purity. We previously demonstrated its safety, immunogenicity, and efficacy against virulent-virus challenge in rodents (4). In that study and another (26), we showed that the vaccine is compatible with a differentiation of infected and vaccinated animals (DIVA) enzyme-linked immunosorbent assay (ELISA). Here, we extend those initial studies to vaccine trials in a commercially important natural RVFV host, merino sheep, including 12 nonpregnant and 29 timed-pregnant animals. Our results demonstrate that ΔNSs-ΔNSm rRVFV is a safe and effective veterinary RVFV vaccine suitable for use in nonpregnant and pregnant animals.

MATERIALS AND METHODS

Biosecurity and general animal husbandry. Adult merino breed sheep were obtained from a commercial source in South Africa and housed in standard livestock hold pens for at least 2 weeks prior to beginning any experimental work. Throughout the experiment, all animals were given ad libitum access to fresh water and sheep mineral blocks and fed a commercial grade pelleted ration appropriate for adult and gestating sheep twice per day. All animal work was conducted under protocols approved by the Deltamune (Pty) Ltd. Animal Use and Ethics Committee; the Republic of South Africa Department of Agriculture, Forestry, and Fisheries; and the U.S. Centers for Disease Control and Prevention (CDC) Institutional Animal Care and Use Committee (IACUC). An initial pilot vaccine safety and immunogenicity dose titration study (pilot dose titration phase) utilized a total of 13 adult sheep. The second study was a larger follow-up safety and efficacy trial in 32 timed-pregnant ewes. During the experiments, all animals were group housed on softwood shaving bedding in a vector-free animal biosafety level 3 (ABSL-3) facility located at the Deltamune laboratory in Pretoria, Republic of South Africa. All laboratorians and animal handlers adhered to international biosafety practices appropriate for BSL-3+/ABSL-3 laboratories and livestock facilities, including full-face, respiratory, and body protection.

Animal vaccination/inoculation procedure and monitoring follow-up. All animals were vaccinated subcutaneously with 1.0 ml of vaccine or vaccine diluent (sterile phosphate-buffered saline [PBS] for sham-vaccinated controls) at various dosages (1.0 × 10^3 to 1.0 × 10^6 PFU) in a wool-free portion of the inguinal area. The general clinical health score of each animal was recorded once daily throughout the study, and rectal temperatures were recorded at the same time of day at least once daily for 30 days p.v. and 21 days postchallenge (p.c.). All animals were assessed at least twice daily by either veterinarians or experienced large-animal technicans, and their general clinical health status was recorded.

RVFV vaccine and virus generation. All in vitro work with live vaccine or virulent challenge RVFV was conducted in a Deltamune BSL-3+/4 facility certified by the South African Department of Agriculture, Forestry, and Fisheries. The ΔNSs-ΔNSm rRVFV vaccine and the rRVFV virulent challenge virus were derived from cDNA plasmids and passed 2 times on Vero E6 cells, the titers were determined, and the virus was subjected to complete genome sequencing prior to use, as previously described (4, 5, 8, 17) (Fig. 1A and B). These recombinant RVFVs were based on the prototype ZH501 strain isolated from a human case during the 1977 to 1979 Egyptian RVF outbreak. The positive immune-marker (enhanced green fluorescent protein [EGFP]) gene that was present in our previously reported work (4) was removed. The ΔNSs-ΔNSm rRVFV vaccine utilized in this study did not contain an EGFP gene or any other positive immune-marker gene.

Viremia testing. All specimens were tested by quantitative reverse transcription-PCR (qRT-PCR) as previously described (6). To account for variation between independent qRT-PCR runs, a standard curve was generated using 10-fold serial dilutions of wild-type RVFV ranging from 1.0 to 0.01 PFU. All specimens were tested by quantitative reverse transcription-PCR (qRT-PCR) as previously described (6). To account for variation between independent qRT-PCR runs, a standard curve was generated using 10-fold serial dilutions of wild-type RVFV ranging from 1.0 to 0.01 PFU. All specimens were tested by quantitative reverse transcription-PCR (qRT-PCR) as previously described (6). To account for variation between independent qRT-PCR runs, a standard curve was generated using 10-fold serial dilutions of wild-type RVFV ranging from 1.0 to 0.01 PFU. In each run, a cutoff cycle threshold (C_T) value representing the limit of detection of 1.0 PFU of infectious virus was set to 35. Any specimen yielding a C_T value between 35 and 40 was further tested for infectious virus by inoculation of Vero E6 cells following standard virus isolation protocols.

RVFV serology. Total anti-RVFV IgM and IgG ELISA testing was completed following standard CDC Viral Special Pathogens Branch diagnostic protocols (7). Briefly, 96-well plates were coated with a final concentration of 1.2.000 of RVFV-infected Vero E6 lysates or mock-infected Vero E6 lysate as a negative background control in PBS and incubated overnight at 4°C. Test sera were added to the plate in 4-fold dilutions starting at 1:100 in 5% skim milk with 0.1% Tween 20. After a 1-hour incubation at 37°C, the plates were washed 3 times with PBS-0.1% Tween 20. Secondary rabbit anti-sheep IgG (KPL) was added at a final concentration of 1.4.000 in 5% skim milk with 0.1% Tween 20 and incubated at 37°C for 1 h. After incubation, the plates were washed 3 times as described previously, and substrate (KPL solution A/solution B) was added following the manufacturer’s recommended protocols. The substrate was incubated at 37°C for 1 h, and absorbance was read at 410 and 490 nm. Cutoff values for each ELISA were established as the mean adjusted summation optical density (SUMOD) of all prebleed animal specimens ± 3 standard deviations. Eighty percent plaque reduction neutralization titers (PRNT_80) were tested using RVFV-infected Vero E6 cells with 1% crystal violet staining as described previously (4).

DIVA ELISA testing. DIVA ELISA, which discriminates between the presence and absence of anti-nucleocapsid (NP), anti-Ns, and anti-NSm IgG antibodies, was also used on a subset of serum specimens collected on prebleed day −1 (all animals), day 78 p.v., or day 37 p.c. We modified our previously reported assay (26) to include a separate analysis of anti-Ns antibodies. Briefly, plates were coated with 200 ng/well each of purified NP, NSs, or NSm and assayed in 2-fold dilutions starting at 1:50 as previously described (26). To assess for nonspecific background reactivity, separate wells were coated with 200 ng/well of purified recombinant Lassa virus G1 protein prepared identically to the RVFV-specific protein antigens. All protein antigens contained a C-terminal histidine tag and were purified from bacterial lysates by standard nickel column chromatography as described previously (26). All background readings were subtracted from anti-RVFV optical density (OD) measurements to yield an adjusted SUMOD value for each protein as described previously (26).

Pilot nonpregnant animal safety and dose escalation study. Twelve healthy nonpregnant female sheep were vaccinated in groups of 4 with increasing doses (1.0 × 10^3, 1.0 × 10^4, and 1.0 × 10^5 PFU) of the ΔNSs-ΔNSm rRVFV vaccine (Fig. 1C). All vaccinated animals were group housed with a sham-vaccinated male animal inoculated with vaccine diluent (sterile PBS) to serve as a direct close-contact sentinel of nonspecific virus spread.

Safety and immunogenicity study using pregnant ewes. A total of 32 timed-pregnant ewes were utilized. The pregnant ewe study had 3 main goals: (i) to determine vaccine safety in the dam and fetus health among the 29 vaccinated
animals during the first 48 days after vaccination (safety 1 arm); (ii) to assess vaccine-related teratology and other adverse events in late gestation and after live birth of the lambs (safety 2 arm), for which 20 of the 29 vaccinated pregnant animals were allowed to progress normally to full-term lamb delivery; and, finally, (iii) to assess vaccine efficacy (vaccine efficacy arm), for which 9 vaccinated animals and the sham-vaccinated controls were challenged intravenously with 1.0\times10^6 PFU of virulent rRVFV at day 80 p.v. (day 122 of gestation) (Fig. 1D). To synchronize gestation, implantable progesterone delivery devices (CIDR, EAZI-breed; Pharmacia Corp., New York, NY) were placed intravaginally for a period of 2 weeks. After device removal, 6 adult rams were introduced to the flock for natural servicing, and all ewes were bred over a period of 48 h. The pregnancy status and stage of fetal development were confirmed 30 days after the rams were introduced by transabdominal ultrasound performed by a veterinary reproduction specialist. Twenty-nine animals were vaccinated at day 42 of gestation with 1.0\times10^4 PFU of the \(\Delta NSs/NSm\) rRVFV vaccine as described above, and 3 pregnant animals were sham vaccinated with sterile PBS to monitor nonspecific vaccine spread from the vaccinated animals.

(i) Safety 1. To ascertain whether the \(\Delta NSs/\Delta NSm\) ZH501 vaccine caused any detectable p.v. viremia and to follow the induction of anti-RVFV-specific total IgM and IgG and neutralizing antibodies (PRNT80), serum and anti-coagulated whole-blood samples were collected from the 29 vaccinated animals and 3 sham-vaccinated controls daily for the first 14 days p.v. and tested by qRT-PCR and virus isolation. At 48 days p.v. (day 90 of gestation), the health and viability of each ewe and fetus(es) were again examined via ultrasound. This ultrasound examination was required to confirm that all fetuses present prior to vaccination remained viable, since in early gestation, fetal materials can be reabsorbed by the mother without any clinically apparent signs of abortion.

(ii) Safety 2. After the initial vaccine safety assessment was completed, the vaccinated animal cohort was subdivided into 2 groups (safety 2 and vaccine efficacy). Twenty animals comprised the safety 2 arm of the experiment and were further bled on days 16, 18, 20, 24, 54, 68, 82, 89, 103, 110, and 117 p.v. to assess the duration of anti-RVFV antibody titers (total IgM and IgG). After delivery, the general health of the lambs from this group was assessed by 3 veterinarians with careful attention to any clinically apparent neurologic or musculoskeletal defects potentially induced by the \(\Delta NSs/\Delta NSm\) rZH501 vaccine. The lambs (n = 22) were euthanized at 1 to 5 days of age. After euthanasia, tissues were collected for qRT-PCR to detect residual vaccine RNA, for histology, and for routine examination by an American College of Veterinary Pathology (ACVP) board-certified veterinary pathologist.

(iii) Vaccine efficacy. To assess the protective efficacy of the \(\Delta NSs/\Delta NSm\) ZH501 vaccine, a group of 9 vaccinated ewes and the 3 sham-inoculated contact control animals were challenged intravenously on day 80 p.v. (gestation day 122)
with rZH501-RVFV (1.0 × 10^6 PFU). To monitor for p.c. viremia and changes in anti-RVFV serology, serum and anti-coagulated whole-blood samples were collected daily for the first 14 days, as well as on days 16, 23, 30, and 37 p.c., and tested by qRT-PCR, virus isolation, total IgM and IgG ELISA, and 3-way DIVA ELISA. The general health of the lambs from animals who did not abort p.c. was assessed by 3 veterinarians, with careful attention to any clinically apparent neurological or musculoskeletal defects potentially induced by the ΔNSs-ΔNSm rZH501 vaccine and virulent challenge virus. The lambs (n = 9) were euthanized at 1 to 5 days of age. Tissues from live-birth lambs and aborted fetuses were collected and analyzed as described above.

**RESULTS**

Reverse genetics rescue, amplification, titration, and complete genome sequencing of the vaccine and virulent rRVFV. Rescue of the ΔNSs-ΔNSm rRVFV vaccine and virulent rZH501-RVFV was 100% successful in 3 independent experimental replicates following our previously reported techniques (4, 5) (Fig. 1A and B). These recombinant viruses were passaged twice on Vero E6 cells at a multiplicity of infection (MOI) of approximately 0.01 with final titers of 5.4 × 10^6 PFU/ml and 4.0 × 10^6 PFU/ml for the vaccine and virulent virus, respectively (Fig. 1B). Complete genome sequencing of each recombinant virus stock revealed that no nucleotide mutations had arisen in any virus stock compared with the original input cDNA plasmid used for virus rescue (data not shown). The vaccine construct used in these studies did not contain a positive immune marker (i.e., an EGFP gene or any other foreign gene).

**Pilot nonpregnant animal safety and dose escalation study.** No detectable febrile response, clinical illness, or inappetance was observed in any of the animals regardless of vaccine dose during the first 30 days p.v. (Fig. 2 and data not shown). No erythema or other signs of localized inflammation could be detected at the vaccination site during the first 72 h p.v. (data not shown), and no vaccination site tissue nodules or abscesses were detected at the end of the 30-day observation period. All sheep, regardless of dose, responded to the vaccination, with robust anti-RVFV IgM responses detectable by 4 to 5 days p.v. and with a subsequent increase in IgG titers detectable by days 8 to 12. A dose-dependent response was observed, with animals receiving the highest vaccine dose (1.0 × 10^5 PFU) mounting more rapid and higher-titer IgM/IgG responses than did animals given the lower doses (Fig. 2). IgG titers ranged from 100 to 400 in the vaccinates (Fig. 2). All vaccinated animals tested negative for RVFV by virus isolation and qRT-PCR regardless of the vaccine dose or time of sample collection during the 30-day observation period (data not shown). No seroconversion or vaccine virus RNA was detected in the group-housed sham-vaccinated control animal (Fig. 2 and data not shown).

**Pregnant ewe safety and efficacy study.** No detectable febrile response was observed during the first 21 days p.v., and no clinical illness or inappetance was detected at any time in the 29 pregnant vaccinated ewes or the 3 sham-vaccinated controls (Fig. 3 and data not shown). No erythema or other signs of localized inflammation could be detected at the vaccination site during the first 72 h p.v., and no vaccination site tissue nodules or abscesses were detected at the end of this phase of the study. No fetal loss was detected during the
ultrasound examination at day 48 p.v. (gestation day 90). All vaccinated animals and sham-vaccinated control animals tested negative for RVFV by virus isolation and qRT-PCR during the first 14 days p.v. (Fig. 3A, bottom). Induction of anti-RVFV IgM was detected approximately 4 to 5 days p.v., with peak titers of at least 6,400, and a subsequent rise of anti-RVFV IgG occurred at days 4 to 7 p.v., with a mean titer of approximately 400 in the vaccinated pregnant animals (Fig. 4A and B). PRNT80 titers began to increase on day 7 p.v., with a robust increase by days 9 and 10. By day 20 p.v., the group mean PRNT80 titer was 190 (Fig. 4C). No seroconversion or vaccine virus RNA was detected at any time in the 3 close-contact sham-vaccinated control animals.

(ii) Safety 2. All 20 ewes delivered at least one healthy, clinically normal lamb (total n = 22) with a normal suckle reflex and mentation appropriate for newborn sheep. Although all fetuses were viable at the ultrasound on day 48 p.v. (gestation day 90), one stillbirth occurred (identification [ID] number 2), followed approximately 20 min later by a normal healthy twin lamb. No evidence of residual vaccine virus RNA or antigen was detectable in multiple organ samples from the stillborn fetus by qRT-PCR, virus isolation, or immunohistochemistry (data not shown). The presence of live twin lambs in the ultrasound at 48 days p.v. and the lack of detectable vaccine virus RNA or antigen in the stillborn lamb tissues indicated that the stillbirth was not attributable to the vaccine. One to 5 days after birth, all lambs were euthanized and a complete necropsy was performed. All lamb tissues (blood, brain, liver, and spleen) tested negative for RVFV by qRT-PCR, virus isolation, and anti-RVFV immunohistochemistry. No abnormalities were detected in histological specimens of the brain (cerebrum, cerebellum, and midbrain), liver, lung, spleen, kidney (ventricular septum and ventricular and atrial wall specimens), adrenal gland, thymus, or mesenteric lymph node. Coronal brain sections at the level of the thalamus exposing the lateral and 3rd ventricles were examined, and no hydranephaly or other central nervous system (CNS) abnormalities were detected (Fig. 5). At the end of this phase (day 120 p.v.), all vaccinated animals had maintained seropositive status, with mean IgG titers of approximately 400 (Fig. 4A).

(iii) Vaccine efficacy. The 9 remaining vaccinated animals and the 3 sham-vaccinated control animals were used in the vaccine efficacy arm of the experiment. All animals were challenged with virulent virus intravenously 80 days p.v. (gestation day 122) with a dose of rRVFV (1.0 × 10^6 PFU) (Fig. 1). This dose and delivery route had previously been determined to cause uniform abortion and moderate to severe disease in adult sheep (data not shown). None of the vaccinated animals developed fever, clinical illness, or inappetance at any time during the 37 days p.c. (Fig. 3B and data not shown). All vaccinated pregnant ewes were protected from RVF-induced abortion and subsequently delivered at least one healthy lamb. No infectious virus or virus RNA could be detected in blood specimens collected daily in the first 14 days p.c. in any of the
vaccinated animals. In contrast, 2/3 of the sham-vaccinated controls developed a fever (\(\text{t} = 40.0^\circ\text{C}\)) that was elevated (\(t\) test; \(P = 0.057\)) compared to vaccinated animals by day 2 postchallenge, and all had rapid increases in RVFV viremia and uniform fetal death confirmed by ultrasound and abortion by 6 to 12 days p.c. (Fig. 3B).

DIVA ELISA. All vaccinated pregnant animals (\(n = 29\)) could be differentiated from the virulent-virus-challenged pregnant animals (\(n = 3\)) using a 3-way recombinant-protein-based DIVA ELISA capable of detecting anti-NSs, -NSm, or -NP IgG antibodies (adapted from reference 26). All vaccinated animals had detectable anti-NP antibodies (average titer, 1:167) but lacked anti-NSs and NSm antibodies (Fig. 4D and data not shown). The 3 sham-vaccinated animals remained seronegative throughout the safety 1 and safety 2 arms (up to day 78 p.v.) of the trial. However, following challenge with virulent virus, these animals rapidly developed robust anti-NP, -NSs, and, in 2 animals, -NSm antibodies (Fig. 4D). Strikingly, no increase in anti-NSs or anti-NSm IgG antibody titers was observed following virulent-virus challenge in the vaccinated animals (\(n = 9\)); a marked increase was detected only in anti-NP antibody titers (Fig. 4D).

DISCUSSION

The impact of RVFV on both livestock and human health is extensive and strongly epidemiologically linked, making RVFV uniquely suitable for a one-health prevention approach through animal vaccination programs. Unfortunately, since the development of the original inactivated and live-attenuated Smithburn (or Smithburn-related) serially passaged vaccines in the 1940s and 1950s, the acceptance of RVFV vaccines for livestock use has been low except in emergency situations. Significant barriers to routine and consistent use of these vac-

FIG. 4. Pregnant animal anti-RVFV serology results. (A) Safety 1 phase total anti-RVFV ELISA testing (\(n = 29\) vaccinated and 3 sham vaccinated) of sheep. After day 82 p.v., the data represent the 20 vaccinated animals that comprised the safety 2 arm of the trial. (B) Total anti-RVFV ELISA results for animals (\(n = 9\) vaccinated and 3 sham vaccinated) that were challenged intravenously with \(1.0 \times 10^6\) PFU of virulent rRVFV at day 82 postvaccination. The days postchallenge are indicated by gray shading. Note the rapid rise in anti-RVFV IgG among vaccinated animals compared with the sham-vaccinated controls. (C) Results of PRNT\(_{80}\) testing during the safety 1 phase (\(n = 29\) vaccinated animals and 3 sham-vaccinated controls). (D) Results of 3-way (NP-NSs-NSm) DIVA ELISA testing at day 54 postvaccination or day 37 postchallenge. The data represent the results from the vaccine efficacy phase of the experiment (\(n = 9\) vaccinated animals and 3 sham-vaccinated control animals). Serum was assayed in 2-fold dilutions from 1:100 to 1:800. The error bars indicate standard deviations of \(\pm 1.0\).
FIG. 5. Gross and histological examination of lamb tissues. (A) Coronal brain sections at the level of the lateral ventricles and hypothalamus obtained from lambs born to the safety 2 phase animals (n = 20 vaccinated ewes and 22 lambs). Note the presence of normal ventricle size and architecture. Also note that slight normal variation can be observed in the color and ventricle shape between the brain sections due to differences in the exact plane of transection. Two brain sections obtained from similarly aged (1- to 5-day-old) normal lambs are shown for comparison (lower right, Cntr1 and Cntr2). (B) Anti-RVFV immunohistochemistry. (Top row) Specimens (×20 magnification) from an aborted RVFV-positive (number 104) fetus depicting liver, cerebrum, and cerebellum (left to right, respectively). Marked diffuse necrosis and antigen staining (red to brown pigment) are apparent in the liver tissue (×40 inset); focal antigen and serum staining can be observed in the cerebrum and cerebellum tissues. (Bottom row) Specimens from a lamb (number 11) born postchallenge to a previously vaccinated ewe; histologically normal and RVFV antigen-negative liver, cerebrum, and cerebellum tissues are shown (left to right, respectively).
cines have included high costs of manufacture, a poorly defined genetic identity, poor efficacy, no capacity to differentiate vaccinated from naturally infected livestock, and, most importantly, the inherent risk of vaccination in pregnant animals due to associated teratogenesis and abortion (9, 10, 19, 31). These factors and others have created a strong reluctance among herdsmen and national governments to implement the sustained vaccination efforts necessary to prevent the massive periodic animal losses and human deaths associated with outbreaks of RVF in areas of endemicity.

To address these concerns, we developed a rationally designed vaccine candidate, ΔNSs-ΔNSm rZH501, based on the complete deletion of the 2 known RVFV virulence factors, the NSs and NSm genes. We have previously reported on the safety of this vaccine in rodents (4) and the potential to discriminate vaccinated from naturally infected animals using a 2-way DIVA ELISA technique based on the virus NP and NSs proteins (26). Here, we extend that work to the first comprehensive examination of the safety and efficacy of this novel RVFV vaccine in healthy nonpregnant and pregnant sheep.

The vaccine caused no p.v. fever, inappetance, or impact on clinical health and was immunogenic at dosages ranging from 1.0 × 10^3 to 1.0 × 10^5 PFU in healthy nonpregnant sheep (Fig. 1 to 3). A large-scale follow-up study in timed-pregnant animals demonstrated that the vaccine was safe for both dam and fetus even when given early in gestation (day 42), when the risk of RVFV vaccine-related teratogenesis is highest (Fig. 3 and 4) (10, 20). Each pregnant animal that received the vaccine delivered at least one healthy lamb free of any clinically apparent physical or neurological defects. Lamb tissues were negative for residual vaccine genome and antigen and free of any vaccine-related histological abnormalities (Fig. 5). The vaccine was also highly immunogenic in pregnant ewes, causing a robust increase in IgM and IgG titers by 4 to 8 days p.v. (Fig. 4A and B).

The vaccine conferred protection from viremia, pyrexia, clinical disease, and abortion in all animals challenged intravenously with 1.0 × 10^6 PFU of virulent rRVFV (Fig. 3 and data not shown). In marked contrast, sham-vaccinated animals developed rapid viremia, pyrexia, and inappetance, and all aborted fetuses within 6 to 12 days p.c. (Fig. 5). Molecular and histological testing of the aborted fetal materials confirmed that the virulent challenge virus was the proximate cause of the observed fetal death and abortion (Fig. 5B, top row, and data not shown).

Furthermore, we detected no spread of the vaccine between vaccinated and nonvaccinated animals even when housed under highly confined laboratory conditions. This lack of vaccine virus spread was likely attributable to the absence of p.v. viremia (Fig. 3). This suggests minimal risk of transmission and dissemination of the vaccine into the environment by blood-feeding vectors, such as mosquitoes. Thus, we conclusively demonstrated that the ΔNSs-ΔNSm rRVFV vaccine can provide a safe and efficacious tool against RVFV in a relevant livestock host, including pregnant animals.

The ΔNSs-ΔNSm rRVFV vaccine was designed to overcome some of the safety limitations of previous serially passaged (Smithburn or MP-12) or naturally occurring mutant (clone 13) vaccines by completely removing the entire open reading frame of the 2 main RVFV virulence factors encoded on two different virus RNA genome segments. In contrast to the MP-12 vaccine strain, which contains 9 amino acid substitutions accumulated over multiple passages in cell culture, the ΔNSs-ΔNSm rRVFV vaccine lacks a total of 264 amino acids (aa) (normally encoded by the S RNA segment) and 129 aa (normally encoded by the M RNA segment), which renders this vaccine much less likely to revert to virulence due to random nucleotide substitutions introduced by viral polymerase errors.

Reassortment of genome RNA segments by members of the family Bunyaviridae during coinfection has been reported in laboratory-derived and field-isolated viruses (2, 8, 12, 30). Importantly, the absence of p.v. viremia or dissemination in ΔNSs-ΔNSm rRVFV-vaccinated animals renders reassortment of cocirculating vaccine and wild-type viruses extremely unlikely. However, even if such a single reassortment event were to occur with the ΔNSs-ΔNSm rRVFV vaccine, the complete deletion of 2 virulence genes on two different RNA segments would ensure that any putative reassortant or recombiant strain was attenuated, as it would still contain at least one major genomic lesion. This is a significant theoretical safety advantage compared to the clone 13 strain vaccine, which carries only an ~70% deletion of the NSs gene (16, 33).

An essential step toward the adoption of an RVFV vaccine for use in areas of nonendemicity is the capacity to differentiate infected animals from vaccinated animals. We show here the utility of a novel 3-way DIVA ELISA (NP, NSs, and NSm) as a platform to provide rapid (~2.5-h), inexpensive, and high-throughput screening of sheep and other livestock without the need for reagents prepared in a high-containment laboratory. This assay showed that, as expected, all vaccinated animals generated antibodies only to the NP structural proteins and none to the deleted NSs and NSm proteins (Fig. 4D and data not shown). Interestingly, we observed variations in the levels of anti-NSs and -NSm antibodies in control virulent-virus-infected animals. In these animals, the titers against the NSs and NSm proteins ranged from 100 to 400 by 37 days p.c. (Fig. 4D). To our knowledge, this is the first time that the immunoreactivity of the NSm protein has been quantitated in any species. From our data, it is clear that a 3-way DIVA assay would likely be more reliable than our originally described 2-way assay (anti-NSs/NSm only) in confirming vaccinated or infected status based on the presence or absence of anti-NSs, anti-NSm, and anti-NP antibodies. Further testing and validation of this new diagnostic platform and adaptation to a field-ready rapid diagnostic tool is under way using a large array of livestock and wildlife specimens collected from areas of endemicity in Africa and Saudi Arabia.

The immunity induced by the ΔNSs-ΔNSm rRVFV vaccine was striking. The body temperature profiles, clinical health, qRT-PCR, and serological assays demonstrated that a robust and sterilizing immune response was generated in the 9 vaccinated animals challenged with 1.0 × 10^6 PFU of virulent RVFV. In these animals, infection with a potentially lethal dose of RVFV was accompanied only by a marked increase in antibody titers (Fig. 4B and D). The 3-way DIVA ELISA clearly showed that this rise was directed specifically to the structural proteins of the inoculated virus, not to the nonstructural NSs and NSm proteins. This finding is significant, since NSs and NSm proteins could only be expressed following vir-
ulent-virus infection and replication. Coupled with the lack of detectable p.c. viremia or fever, this finding strongly suggests that a sterilizing immune response was generated by the ΔNSs-ΔNSm RVFV vaccine.

Control programs for RVFV provide an ideal test case for the merits of a one-health approach to zoonosis prevention. A DIVA-compatible vaccine platform would be clearly beneficial to livestock growers by directly improving animal health and economically by allowing the control of outbreaks while respecting international trade regulations requiring serological proof of disease-free herd status. Our data presented here demonstrate that the ΔNSs-ΔNSm RVFV vaccine could be utilized to fulfill these needs as a safe and effective tool to combat the significant impacts of RVFV on humans and animals.

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