Rift Valley Fever Virus Lacking the NSs and NSm Genes Is Highly Attenuated, Confers Protective Immunity from Virulent Virus Challenge, and Allows for Differential Identification of Infected and Vaccinated Animals

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Rift Valley fever (RVF) virus is a mosquito-borne human and veterinary pathogen associated with large outbreaks of severe disease throughout Africa and more recently the Arabian peninsula. Infection of livestock can result in sweeping “abortion storms” and high mortality among young animals. Human infection results in self-limiting febrile disease that in ~1 to 2% of patients progresses to more serious complications including hepatitis, encephalitis, and retinitis or a hemorrhagic syndrome with high fatality. The virus S segment-encoded NSs and NSm proteins are important virulence factors. The development of safe, effective vaccines and tools to screen and evaluate antiviral compounds is critical for future control strategies. Here, we report the successful reverse genetics generation of multiple recombinant enhanced green fluorescent protein-tagged RVF viruses containing either the full-length, complete virus genome or precise deletions of the NSs gene alone or the NSs/NSm genes in combination, thus creating attenuating deletions on multiple virus genome segments. These viruses were highly attenuated, with no detectable viremia or precise illness observed with high challenge dosages (1.0 × 10^3 PFU) in the rat lethal disease model. A single-dose immunization regimen induced robust anti-RVF virus immunoglobulin G antibodies (titer, ~1:6,400) by day 26 postvaccination. All vaccinated animals that were subsequently challenged with a high dose of virulent RVF virus survived infection and could be serologically differentiated from naïve, experimentally infected animals by the lack of NSs antibodies. These rationally designed marker RVF vaccine viruses will be useful tools for in vitro screening of therapeutic compounds and will provide a basis for further development of RVF virus marker vaccines for use in endemic regions or following the natural or intentional introduction of the virus into previously unaffected areas.

The Rift Valley fever (RVF) virus (family Bunyaviridae, genus Phlebovirus) is a mosquito-borne pathogen of both livestock and humans, found throughout Africa and, more recently, the Arabian peninsula. Historically, RVF virus has been the cause of either low-level endemic activity or large explosive epizootics/epidemics of severe disease throughout its range (19, 28, 46, 61). RVF virus disease outbreaks are characterized by economically disastrous “abortion storms” and a newborn animal mortality approaching 100% among livestock, especially for sheep and cattle (17, 18, 47). Human infections typically occur either from infected mosquito bites or as the result of percutaneous/aerosol exposure during the slaughter of infected animals or via contact with aborted fetal materials. Human RVF virus disease is primarily a self-limiting febrile illness that in a small percentage (~1 to 2%) of cases, can progress to more serious and potentially lethal complications including hepatitis, delayed onset encephalitis, retinitis, blindness, or a hemorrhagic syndrome with a hospitalized case fatality rate of 10 to 20% (32, 33, 35). Excessively heavy rainfall in semi-arid regions often precedes large periodic outbreaks of RVF virus activity, allowing for the abundant emergence of transovarially infected Aedes spp. mosquitoes and the subsequent initiation of an outbreak by transmission of virus to livestock and humans via infected mosquito feeding (30, 53). The association with abnormally heavy rains provides some ability to predict the periods and the regions of high disease risk, thus providing a potential window of opportunity for targeted vaccination programs, if a safe, inexpensive and highly efficacious single dose vaccine were available.

The abilities of the RVF virus to cross international and geographic boundaries and to strain veterinary and public health infrastructures are well documented. In 1977, RVF virus was reported for the first time north of the Sahara desert, where an extremely large outbreak affecting more than 200,000 people occurred along the Nile River basin in Egypt (34). Approximately 10 years later, in 1987, a large outbreak occurred in western Africa, along the border of Mauritania and Senegal, affecting an estimated 89,000 individuals (28). Later, the virus was isolated for the first time outside of Africa (across the Red Sea) in Saudi Arabia and Yemen and was found to be...
the cause of a large epizootic/epidemic in 2000, with an estimated 2000 human cases and 245 deaths (14, 15, 51). Most recently, in late 2006 and early 2007, following heavy rainfall in eastern Africa, RVF virus emerged as the cause of a widespread outbreak that eventually resulted in a total of 1,062 reported human cases and 315 deaths and was associated with substantial economic losses among livestock in southern Somalia, Kenya, and northern Tanzania (13). The ability of RVF virus to cause explosive “virgin soil” outbreaks in previously unaffected regions, accompanied by high morbidity and mortality during RVF epizootics/epidemics, highlights the importance of developing high-throughput screening tools for potential antiviral therapeutic agents and safe and efficacious vaccines for this significant veterinary and public health threat.

Like other members of the genus *Phlebovirus*, the negative sense single-stranded RNA genome of the RVF virus is tripartite (50). The small (S) segment (~1.6 kb) encodes, in an ambisense fashion, the virus nucleoprotein (NP) in the genomic (negative-sense [−]) orientation and the nonstructural (NSs) protein in the antigenomic (positive-sense [+]) orientation (1, 50). The medium (M) segment (~3.8 kb) contains at least four nested proteins in a single open reading frame (ORF): the two structural glycoproteins Gn and Gc and two nonstructural proteins, the 14-kDa NSm and the 78-kDa NSm-Gn fusion protein (20, 21, 50). The large (L) segment (~6.4 kb) contains the viral RNA-dependent RNA polymerase (50).

Both nonstructural genes (NSs and NSm) have been found to function as virus virulence factors and determinants of mammalian host pathogenesis (7, 58, 60). NSs has been shown to mediate the pan-downregulation of mRNA production by the inhibition of RNA polymerase II activity (6, 29). Via this mechanism, the NSs protein performs a critical role in mammalian host pathogenesis by indirectly disrupting the host cell antiviral response (10, 40, 58).

The nonstructural gene located on the RVF virus M segment (NSm) was recently found to be dispensable for efficient RVF virus growth in both interferon-competent and interferon-deficient cell cultures (20, 59). However, further work utilizing a highly sensitive animal model revealed that recombinant RVF virus lacking the entire NSm coding region (rRVF-ΔNSm) was attenuated yet retained the ability to cause either acute onset lethal hepatic necrosis or delayed onset lethal neurologic disease in a minority (44%) of animals (7). Recent work (60) has demonstrated that NSm functions as a virus virulence factor by suppressing the host cell apoptotic pathway following infection.

In the current study, we exploit the recent findings of NSs and NSm involvement in virus pathogenicity to create rationally designed RVF virus vaccine candidates that are highly attenuated, immunogenic, and contain precise molecular markers, allowing for the differentiation of naturally infected and vaccinated animals (DIVA). We hypothesized that reverse genetics-derived recombinant RVF virus containing complete deletions of the major virus virulence factors NSs and NSm would be highly attenuated in vivo and that these recombinant viruses would induce robust anti-RVF virus antibody responses, providing protection from virulent virus challenge, and would allow for the assessment of the vaccination status of animals on the basis of NSs/NP serology. Secondarily, by incorporating a reporter moiety into these viruses, we sought to simultaneously develop live virus research tools that might be useful for the rapid screening of antiviral therapeutic compounds.

We report here the results of in vitro testing of the utility and stability of recombinant RVF viruses containing the insertion of the reporter moiety enhanced green fluorescent protein (eGFP) for use in antiviral compound screening assays and the results of the in vivo safety, immunogenicity, and protective efficacy of two potential vaccine candidates lacking the entire NSs and NSm genes. These results comprise the first report of an eGFP-tagged virus containing the complete RVF virus genome and the first in vivo studies of a reverse genetics-generated recombinant RVF virus vaccine candidate containing precise deletions of complete virus genes with known roles in virulence. These recombinant viruses will provide a basis for the development of safe and effective human or veterinary vaccines for this significant veterinary and public health threat.

**MATERIALS AND METHODS**

**RVF virus and biosafety.** All work with infectious RVF virus (wild type or recombinant strain) was conducted within the CDC biosafety level 4 (BSL-4) laboratory. Low-passage (FRhL, two passages, and Vero E6, two passages) working stocks of the wild-type strain ZH501, isolated originally from an Egyptian case of fatal human infection in 1977, were used in this work as challenge viruses and were prepared by passage on Vero E6 cell monolayers. The complete genome sequences of the S, M, and L segments of the wild-type RVF virus strain ZH501 used in this work can be found under GenBank accession numbers DQ380149, DQ380200, and DQ375406, respectively.

**Construction of the ΔNSs/eGFP deletion/replacement plasmid.** To generate plasmids containing the complete deletion of the NSs ORF and replacement with the eGFP ORF, we first amplified the eGFP ORF by PCR with forward and reverse primers containing the KpnI (GGTACC) and the BglII (AGATCT) restriction sites, respectively. Utilizing the full-length RVF-S segment plasmid, we conducted a strand-specific PCR with primers designed to contain the KpnI and BglII restriction sites, annealing immediately upstream to the NSs start codon and immediately downstream of the NSs stop codon. The resulting PCR fragment contained a complete deletion of the NSs ORF flanked by the KpnI and BglII restriction sites. The eGFP and RVF-S plasmid amplicons were gel purified and ligated following standard molecular biology techniques. The resulting plasmid (rRVFS-ΔNSs/eGFP) contained a complete in-frame replacement of the NSs ORF with eGFP.

**Construction of plasmids containing an NSs-eGFP fusion protein.** Using a second set of plasmid constructions, we sought to generate rRVF viruses containing complete full-length genomes and an inserted full-length eGFP ORF. Two constructions were made, containing in-frame fusions of the C terminus of NSs with the eGFP ORF, separated by amino acid linker moieties of various lengths. In the first construction, the full-length RVF-S segment plasmid backbone was modified using site-directed mutagenesis PCR (QuickChange; Stratagene) with overlapping primers containing a deletion/replacement of the NSs stop codon with a linker section containing nucleotides encoding three alanine residues and the KpnI restriction site. Following this step, both this amplicon and the prRVFS-ΔNSs/eGFP plasmid were digested with KpnI. The resulting restriction enzyme fragments were gel purified and ligated to generate the final prRVFS-NSs(A3)ΔeGFP construction. A second NSs-eGFP fusion peptide construction was created to increase the length of the eGFP linker moiety and to eliminate the KpnI restriction site contained in the previous fusion peptide construction. To accomplish this, we again employed site-directed mutagenesis (QuickChange; Stratagene) starting with the prRVFS-NSs(A3)ΔeGFP backbone and overlapping primers containing the remote cutter restriction enzyme BsmBI and nucleotides encoding 10 alanine residues. Following PCR amplification, restriction enzyme digestion, and religation, the resulting construction contained a perfect in-frame fusion of the C terminus of NSs with the N terminus of the eGFP ORFs in the context of the complete full-length RVF virus S segment genome, preserving the spacing and nucleotide sequence of both the NP and the NSs transcription termination signals.

**Generation and in vitro testing of recombinant RVF viruses.** In all cases, the rescue of recombinant viruses was accomplished as described previously, using cDNA plasmids encoding the virulent RVF virus ZH501 strain (7). The basic
design and construction of the full-length plasmids containing inserts of the complete S, M, and L segments and plasmids containing deletions of the NSm gene were reported previously (7, 20). Briefly, antigenic sense plasmids representing the three genomic segments were transfected in 1-μg quantities with LT-1 (Mirus) at a ratio of 6:1 and transferred onto subconfluent (60 to 70%) monolayers of BSR-T7/5 cells stably expressing the T7 polymerase. These cells were a kind gift from Klaus Conzelmann (Max-von-Pettenkofer-Institut, Munich, Germany). Four or five days posttransfection, the cell supernatant was clarified by low-speed centrifugation and passaged twice on confluent monolayers of Vero E6 cells. After passage and prior to use in subsequent experiments, the exact complete genome sequence of each rescued recombinant virus was confirmed by techniques described previously (9).

**Infected live cell (direct) or fixed cell (indirect) fluorescent-antibody detection of RVF virus NSs and eGFP proteins.** Vero E6 cells were seeded on glass coverslips and infected at a multiplicity of infection of approximately 1.0 with either rZH501-ΔNSs:GFP, rZH501-ΔNSs:GFP-ΔNSm, rZH501-NSm(Ala)18, or rZH501-NSs(Ala)26. At 24 h postinfection, cells were visualized directly by inverted UV microscopy (live cell detection) or were fixed in 10% formalin overnight. Following fixation, infected cells were gamma irradiated (5.0 × 10³ rad) to inactivate any residual virus activity. After cells were inactivated, they were permeabilized (using 0.05% Triton X-100) and incubated with monoclonal antibodies specific for either RVF virus NSs or eGFP protein, following standard techniques.

**Animal immunization and infection.** A total of 66 female Wistar-Furth (WF/Nsd) (Harlan) rats, 6 to 8 weeks of age (∼160 g), were used in this study and housed in microisolation pens and provided with food and water ad libitum. All pens were kept in HEPA filtration racks following standard barrier care techniques. All animals were housed in a BSL-2 laboratory, following standard protocols as described previously (56) for noncellular lysis buffer (Applied Biosystems) for decontamination and transfer to vaccine-induced viremia. This whole-blood sample was placed directly into a 2-ml sample tube, as described above. An additional 20 rats were divided into two dosage groups of 10 animals each and inoculated SQ with either 1.0 × 10⁶ PFU of the virulent wild-type RVF virus strain ZH501 or 200 μl of sterile saline. A total of eight animals served as sham-inoculated animals (three in the pilot study and five in the vaccination/challenge study) and were administered sterile saline only. All animals were examined daily postinoculation for signs of clinical illness, weight loss, and respiratory distress. Animals were euthanized by either in excess or following disturbances or peritoneal inoculation with 10% isoflurane and then euthanized using pentobarbital sodium and phenytoin sodium solution (Schering-Plough) following standard techniques. All procedures were approved by the CDC Institutional Animal Care and Use Committee and the CDC Institutional Biosafety Committee.

**Pilot in vivo immunogenicity and safety study.** A total of 18 rats received 1.0 × 10⁶ PFU SQ of either rZH501-ΔNSs:GFP (nine animals) or rZH501-ΔNSs:GFP-ΔNSm (nine animals), with three animals serving as sham-inoculated controls (receiving sterile saline only). After the rats were vaccinated, small (∼25 μl) samples of whole blood were obtained via the tail vein on days 1 to 4 to detect vaccine-induced viremia. This whole-blood sample was placed directly into a 2-ml sample tube, as described above. An additional 20 rats were divided into two dosage groups of 10 animals each and inoculated SQ with either 1.0 × 10⁶ or 1.0 × 10⁵ PFU of the rZH501-ΔNSs:GFP virus, as described above. An additional 20 rats were divided into two dosage groups of 10 animals each and inoculated SQ with either 1.0 × 10⁶ or 1.0 × 10⁵ PFU of the rZH501-ΔNSs:GFP-ΔNSm virus, as described above. A total of five animals served as sham-inoculated (sterile saline-only) controls. On days 2, 4, and 7 postvaccination, a small blood sample (∼25 μl) was collected from the rats' tail veins and added directly to a 2× noncellular lysis buffer (Applied Biosystems) for decontamination and transfer to a BSL-2 laboratory, following standard protocols as described previously (56) for subsequent RNA extraction and RVF virus-specific quantitative reverse transcription-PCR (q-RT-PCR). At day 21 postvaccination, all animals were anesthetized using isoflurane, serum samples were collected for the determination of total anti-RVF virus immunoglobulin G (IgG) titers, and then the animals were euthanized using pentobarbital sodium-phenytoin sodium solution (Schering-Plough). Following confirmation of the exact molecular sequence, each plasmid was transfected separately on Vero E6 cells grown on glass coverslips in 1-μg quantities at a 6:1 ratio with a lipofectamine solution (LT-1; Mirus). Following a 48-h incubation, transfected cells expressing either the NP or the NSs protein were fixed and stained with a 1- to 2-ml nutrient agarose overlay (minimum essential medium [1% fetal bovine serum, 2% diaminodiphenylindole (DAPI) counterstaining. To confirm the presence of anti-RVF virus NP and NSs antibodies among the naturally infected animals, serum samples collected from goats in Saudi Arabia during the outbreak in 2000 were tested essentially as described above with anti-goat-specific total IgG (Alexa Fluor 488 nm; fluorescein isothiocyanate; Molecular Probes/Invitrogen) antibody. Intracellular localization of NSs protein and rat antibody was visualized by the secondary adsorption of an Alexa Fluor 594-nm (22) anti-rat total IgG (Molecular Probes/Invitrogen) antibody. Intracellular localization of NSs protein and rat antibody was confirmed by 4',6'-diamidino-2-phenylindole (DAPI) counterstaining. To confirm the presence of anti-RVF virus NP and NSs antibodies among the naturally infected animals, serum samples collected from goats in Saudi Arabia during the outbreak in 2000 were tested essentially as described above with anti-goat-specific total IgG (Alexa Fluor 488 nm; fluorescein isothiocyanate; Molecular Probes/Invitrogen).

**Anti-RVF virus total IgG enzyme-linked immunosorbent assay.** Determination of anti-RVF virus IgG titers from vaccinated and control rats was performed essentially as described previously (32), with the following modifications necessary for rat specimens. Standard 96-well microtiter plates were coated overnight at 4°C with 100 μl of gamma-irradiated RVF virus-infected Vero E6 cell lysate diluted 1:2,000 (in 0.01 M phosphate-buffered saline [PBS; pH 7.2] or similarly diluted gamma-irradiated, uninfected Vero E6 cell lysate) to serve as adsorption controls. Plates were washed three times (PBS-0.01% Tween-20), and 100-μl duplicate samples of rat sera were diluted from 1:100 to 1:16,400 in fourfold dilutions in skim milk serum diluent and adsorbed for 1 h at 37°C. Plates were washed three times (PBS-0.01% Tween-20), and 100 μl of goat anti-rat IgG horseradish peroxidase-conjugated antibody (KPL, Gaithersburg, MD) diluted 1:2,500 was added for 1 h at 37°C. After another three washes, 100 μl of ABTS substrate (KPL, Gaithersburg, MD) was added and incubated for 30 min at 37°C. Plates were read at 410 nm, with an absorbance correction of 490 nm for plate imperfections. The absorbance values of the 1:100, 1:400, 1:1,600, and 1:6,400 dilutions were added and constituted a sum of the optical densities (SumOD) value for each specimen. The background nonspecific adsorption of each animal's serum on negative control Vero E6 cells was subtracted from the calculated SumOD value obtained from the experiment. The final serum dilution yielding an adjusted SumOD of ≥0.20. A cutoff value for determining positive versus negative samples was established as the mean sample-adjusted SumOD ± three standard deviations obtained from the five sham-inoculated control animals.

**Anti-RVF virus NSs and NP differential indirect fluorescent-antibody assays.** Plaque retraction of the RVF virus strain ZH501, in which the NSs-specific antibody responses were generated following techniques described previously (41). Briefly, oligonucleotide primers were designed to anneal within the NP or the NSs ORF with the addition of a SacI and BglII restriction site for cloning into a polymerase II-based expression plasmid, pCAGGS. The resulting PCR amplicons were agarose gel purified, digested with SacI and BglII, and ligated between the corresponding restriction sites of the pCAGGS vector. Prior to their use, the resulting clones, pC-NSP and pC-NSs, were sequenced using standard techniques. Following confirmation of the exact molecular sequence, each plasmid was transfected separately on Vero E6 cells grown on glass coverslips in 1-μg quantities at a 6:1 ratio with a lipofectamine solution (LT-1; Mirus). Following a 48-h incubation, transfected cells expressing either the NP or the NSs protein were fixed and stained with a 1- to 2-ml nutrient agarose overlay (minimum essential medium [1% fetal bovine serum, 2% diaminodiphenylindole (DAPI) counterstaining. To confirm the presence of anti-RVF virus NP and NSs antibodies among the naturally infected animals, serum samples collected from goats in Saudi Arabia during the outbreak in 2000 were tested essentially as described above with anti-goat-specific total IgG (Alexa Fluor 488 nm; fluorescein isothiocyanate; Molecular Probes/Invitrogen) antibody. Intracellular localization of NSs protein and rat antibody was confirmed by 4',6'-diamidino-2-phenylindole (DAPI) counterstaining. To confirm the presence of anti-RVF virus NP and NSs antibodies among the naturally infected animals, serum samples collected from goats in Saudi Arabia during the outbreak in 2000 were tested essentially as described above with anti-goat-specific total IgG (Alexa Fluor 488 nm; fluorescein isothiocyanate; Molecular Probes/Invitrogen).
formalin overnight. Following fixation, the agarose overlay was removed, and the plates’ surfaces were decontaminated and gamma irradiated (2.0 × 10⁶ rad) following standard BSL-4 safety procedures. After inactivation, the cell monolayer was stained with 1% crystal violet in PBS, and plaques were enumerated.

The calculated PRNT₅₀ corresponded to the reciprocal titer of the last dilution, resulting in a 50% reduction in the number of plaques compared to that of controls.

**RVF virus-specific q-RT-PCR.** Whole rat blood was assayed for the presence and quantity of RVF virus-specific RNA following protocols described previously (8). Quantification of total serum RVF virus RNA was calculated directly via interpolation from a standard curve generated from serial dilutions of stock RVF virus strain ZH501, of a known titer, in whole rat blood extracted and processed in a manner identical to that of each experimental replicate q-RT-PCR run. Briefly, 25 μl of whole rat blood (either from vaccinated/challenged animals or from stock virus serial dilutions) was added to 300 μl of 2× noncellular lysis buffer (Applied Biosystems), and total RNA was extracted (ABI 6100 nucleic acid workstation; Applied Biosystems). After RNA was extracted, cDNA was generated by random hexamer priming (high-capacity cDNA kit; Applied Biosystems), followed by RVF virus-specific q-PCR (Universal q-PCR master mixture; Applied Biosystems), using the protocol, the primer/probe combination, and the thermocycling conditions outlined previously (8). Results are reported as RVF virus PFU equivalents (eq)/ml of rat blood.

**Statistical analyses.** For all calculations, XLSTAT analysis software (AddinSoft) was utilized. Kaplan-Meier analyses were completed with log-rank and Wilcoxon tests of significance with an α-level setting of 0.05. Analyses of SumOD and viremia were completed utilizing a one-way analysis of variance and the thermocycling conditions outlined previously (8). Results are reported as RVF virus PFU equivalents (eq)/ml of rat blood.

**RESULTS**

**Generation and in vitro stability testing of rRVF viruses.** As described previously (7), the rescue of all recombinant viruses used in this study was accomplished by transfection of three (full-length or deletion mutant) antigenomic sense plasmids representing each of the three virus RNA segments, without the requirement for supporting expression plasmids encoding virus structural proteins. Multiple recombinant RVF (rRVF) viruses were generated, containing an inserion of the reporter molecule eGFP into the virus S segment. From among these viruses, two rRVF viruses were rescued, containing an intramolecular fusion of the C terminus of the NSs protein with the N terminus of eGFP, separated by a peptide linker of either 3 or 10 alanine residues [rZH501-NSs(Ala)₃GFP and rZH501-NSs(Ala)₁₀GFP, respectively; Fig. 1A and B]. Two vaccine candidate viruses were created containing deletions of either the NSs gene alone or the NSs/NSm genes in combination (rZH501-ΔNSs:GFP and rZH501-ΔNSs:GFP-ΔNSm, respectively). In both of these viruses, the NSs gene was replaced by the reporter molecule eGFP, preserving the native S segment ambisense RNA orientation (Fig. 1A and B). Both rRVF virus vaccine candidate viruses were rescued upon the first attempt and grew to high titers, routinely exceeding 1.0 × 10⁶ PFU eq/ml, in Vero E6 cell culture, resulting in complete monolayer lysis (data not shown). Following passage on Vero E6 cells, a cytoplasmic GFP signal was first observed approximately 10 to 12 h postinfection and appeared to spread rapidly throughout the cell monolayer prior to the first signs of extensive cytopathic effect and plaque formation (data not shown). Recombinant virus containing NSs-GFP fusion peptides [rZH501-NSs(Ala)₃GFP and rZH501-NSs(Ala)₁₀GFP] were also rescued on the first attempt but were found to grow to slightly lower titers (∼5.0 × 10⁵ PFU eq/ml). As expected, the NSs-GFP fusion protein was first localized in the cytoplasm of infected cells, followed by perinuclear accumulation and eventual intranuclear migration, followed by the formation of filamentous structures at 12 to 18 h postinfection (Fig. 1B and C).

The stability of the eGFP reporter gene in all recombinant viruses reported here was monitored for 15 serial passages (1:100 dilution between each passage) in Vero E6 cells, during which time no decrease in the stability of eGFP expression was observed, with all infected cells expressing a robust amount of eGFP protein similar to that seen in early passages (Fig. 1B).

**Pilot in vivo immunogenicity and safety study.** To gain a primary assessment of the relative in vivo characteristics of the rZH501-ΔNSs:GFP and rZH501-ΔNSs:GFP-ΔNSm recombinant viruses, groups of nine rats were inoculated with each vaccine candidate at a dose of 1.0 × 10⁵ PFU (Table 1). Animals were monitored daily for signs of clinical illness and weight loss. At no time postvaccination did any animal show signs of clinical illness, and all experienced average daily weight changes equal to those of the sham-inoculated controls of approximately 0 to 5 g (data not shown). All vaccinated rats were bled (~25 μl) on days 1 to 4 postinoculation to determine the titer of vaccine-induced viremia. Surprisingly, using a highly sensitive q-RT-PCR assay, no animal, at any time point analyzed postvaccination, developed a detectable viremia (Table 1). All animals were euthanized at day 21 postvaccination, and anti-RVF virus total IgG antibody titers were evaluated. Testing revealed that the mean SumOD (± standard error of the mean [SEM]) for all animals vaccinated with the rZH501-ΔNSs:GFP virus was 2.14 ± 0.12, which corresponded to end-point titers of 1:1,600 in 66% (6/9) of the animals, and the remaining 33% (3/9) had titers equal to 1:400 (Fig. 2 and Table 1). Among animals receiving the rZH501-ΔNSs:GFP-ΔNSm virus, the mean SumOD ± SEM was 1.24 ± 0.06, with 89% (8/9) of the animals developing end-point dilution titers equal to 1:400 (Fig. 2 and Table 1). Unfortunately, due to technical issues, serum was not obtained from the remaining animal in this group. As expected, all sham-inoculated animals were negative for detectable levels of anti-RVF virus total IgG antibody (Table 1; mean SumOD of 0.08 ± 0.06).

All vaccinated animals in the rZH501-ΔNSs:GFP and rZH501-ΔNSs:GFP-ΔNSm virus groups developed statistically higher mean anti-RVF virus total IgG SumOD values than the nonvaccinated controls (P < 0.001 and P = 0.003, respectively; Table 1). Interestingly, animals in the rZH501-ΔNSs:GFP virus group developed significantly higher mean SumOD values than animals given the rZH501-ΔNSs:GFP-ΔNSm vaccine (P = 0.004; Table 1). The PRNT₅₀ testing was completed with a subset (four animals) chosen randomly from each vaccine group, with two sham-inoculated animals serving as controls. Results were encouraging, with mean PRNT₅₀ of 1:1,480 (rZH501-ΔNSs:GFP) and 1:280 (rZH501-ΔNSs:GFP-ΔNSm), and with sham-inoculated control animal titers of ≤1:10 (Table 1).
ence of detectable viremia. As was observed for the pilot study, at no time did any animal develop detectable vaccine viremia (Table 1). Additionally, no clinical illness was observed for any of the vaccinated animals, and all animals experienced weight gains of approximately 1 to 5 g per day, similar to those of the sham-inoculated controls. At day 26 postvaccination, serum samples were obtained to determine the levels of total anti-RVF virus IgG, PRNT<sub>50</sub>, and anti-NP/anti-NSs protein-specific antibody production prior to the subsequent challenge on day 28. Encouragingly, all animals, regardless of recombinant virus type or dose, developed high-titered total anti-RVF virus IgG antibody (Fig. 2 and Table 1). Among rats receiving the rZH501-ΔNSs:GFP virus, the mean Sum<sub>OD</sub> ± SEM was 4.10 ± 0.12 (for the 1.0 × 10<sup>3</sup> dose group) and 4.79 ± 0.11 (for the 1.0 × 10<sup>4</sup> dose group), which corresponded to 85% (17/20) of animals developing anti-RVF virus IgG end-point titers of 1:6,400. The remaining three animals in the rZH501-ΔNSs:GFP virus group developed end-point titers equal to 1:1,600. In animals receiving the rZH501-ΔNSs:GFP-ΔNSm virus, the mean Sum<sub>OD</sub> ± SEM was 3.94 ± 0.12 (the 10<sup>3</sup> dose group) and 4.54 ± 0.11 (the 10<sup>4</sup> dose group), which corresponded to 75% (15/20) of the developing anti-RVF virus IgG end-point titers of 1:6,400 and with the remaining 25% (5/20) attaining end-point titers of 1:1,600. As was observed for the pilot study, all animals vaccinated with either rZH501-ΔNSs:GFP or rZH501-ΔNSs:GFP-ΔNSm virus, regardless of the dose, developed statistically significant higher mean Sum<sub>OD</sub> values than the sham-inoculated control animals (P values for all were <0.05) (Fig. 2; Table 1). Likewise, PRNT<sub>50</sub> were found to be elevated above those of the sham-inoculated controls, among animals...
inoculated with the rZH501-ΔNSs:GFP virus, with mean titers of 1:640 and 1:7,040 in the 1×10^3 and the 1×10^4 dose groups, respectively (Table 1). The mean PRNT_{50} values among animals vaccinated with the rZH501-ΔNSs:GFP-ΔNSm virus were found to be similar, with mean titers of 1:1,120 and 1:640 in the 1×10^3 and the 1×10^4 dose groups, respectively (Table 1).

(ii) Challenge phase. On day 28 postvaccination, all rats were challenged with a known lethal dose (1×10^3 PFU) of virulent wild-type strain ZH501. All animals were monitored

### TABLE 1. Summary results from the pilot study and the follow-up challenge study

<table>
<thead>
<tr>
<th>Study</th>
<th>Inoculum</th>
<th>No. of animals</th>
<th>Vaccine dose (PFU)</th>
<th>Anti-RVF virus IgG mean Sum_{OD} ± SEM</th>
<th>Mean PRNT_{50} ± SEM</th>
<th>Postvaccination viremia (PFU eq/ml whole blood) at days:</th>
<th>RVF viremia peak titer postchallenge</th>
<th>No. of animals surviving challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pilot study (day 21 postimmunization)</td>
<td>rRVF-ΔNSs:GFP</td>
<td>9</td>
<td>1×10^3</td>
<td>2.14±0.12§</td>
<td>1:1,480±631</td>
<td>0.0</td>
<td>0.0* (10)</td>
<td>10/10*</td>
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<tr>
<td></td>
<td>rRVF-ΔNSs:GFP-ΔNSm</td>
<td>9</td>
<td>1×10^3</td>
<td>1.24±0.06§</td>
<td>1:280±120</td>
<td>0.0</td>
<td>0.0* (9)</td>
<td>10/10*</td>
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<td>0</td>
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<td>1:10±0.00</td>
<td>0.0</td>
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</tr>
<tr>
<td>Challenge study (day 26 postimmunization)</td>
<td>rRVF-ΔNSs:GFP</td>
<td>10</td>
<td>1×10^3</td>
<td>4.10±0.12*</td>
<td>1:640±0.0</td>
<td>0.0</td>
<td>0.0* (9)</td>
<td>10/10*</td>
</tr>
<tr>
<td></td>
<td>rRVF-ΔNSs:GFP</td>
<td>10</td>
<td>1×10^3</td>
<td>4.79±0.11*</td>
<td>1:7,040±3,200</td>
<td>0.0</td>
<td>1.5×10^1 (1)</td>
<td>3/10*</td>
</tr>
<tr>
<td></td>
<td>rRVF-ΔNSs:GFP-ΔNSm</td>
<td>10</td>
<td>1×10^3</td>
<td>3.94±0.12*</td>
<td>1:1,120±733</td>
<td>0.0</td>
<td>0.0* (9)</td>
<td>10/10*</td>
</tr>
<tr>
<td></td>
<td>rRVF-ΔNSs:GFP-ΔNSm</td>
<td>10</td>
<td>1×10^4</td>
<td>4.54±0.13*</td>
<td>1:640±0.0</td>
<td>0.0</td>
<td>1.1×10^2 (1)</td>
<td>3/10*</td>
</tr>
<tr>
<td></td>
<td>Negative control</td>
<td>5</td>
<td>0</td>
<td>−0.02±0.09</td>
<td>1:5±2.9</td>
<td>0.0</td>
<td>7.0×10^1 (1)</td>
<td>2/5</td>
</tr>
</tbody>
</table>

*Summary data from the pilot and follow-up challenge studies. Results with significant differences (P value of <0.05) between the vaccinated and sham-inoculated animal group values (mean viremia postchallenge, total anti-RVF IgG SUM_{OD}, PRNT_{50} titer, and mean survival time) are indicated by an asterisk. Significant differences between ΔNSs and ΔNSs-ΔNSm mean total anti-RVF virus IgG SUM_{OD} values were observed during the pilot study (day 21 postvaccination) and are indicated with a section (§) symbol. Note that in the larger follow-up challenge study (day 26 postvaccination), these differences were found to be insignificant.

FIG. 2. Results of anti-RVF virus total IgG adjusted SUM_{OD} enzyme-linked immunosorbent assay of all vaccinated (40) and sham-inoculated (5) control animals on day 26 postimmunization. Note the lack of anti-RVF virus total IgG among sham-inoculated controls compared with that of vaccinated animal groups. A positive/negative cutoff value was established as the mean ± three standard deviations of the sham-inoculated SUM_{OD} values (open circles-dashed line). Significant differences between the vaccinated and the control groups (P value of <0.05) are indicated by a “§” symbol. No significant differences were found among vaccinated groups, regardless of the dose or the vaccine used. Mean SUM_{OD} values are shown as a heavy black line; error bars indicate the mean ± 2 standard deviations.
daily for signs of clinical illness and weight loss/gain for 42 days postchallenge. At no time postchallenge (days 1 to 42) did any rat that received prior vaccination with either the rZH501-
\Delta NSs:GFP or the rZH501-\Delta NSs:GFP-\Delta NSm virus develop clinically detectable illness (ruffled fur, hunched posture, or lethargy). On approximately day 2 postchallenge, a majority of animals did suffer slight reductions, 1 to 5 g, in total body weight, which was regained by day 5 postchallenge. Rat whole blood was obtained on days 2, 3, 4, and 7 postchallenge and assayed for the presence of RVF virus RNA by a highly sensitive q-RT-PCR assay (8). Following the challenge, low-level viremia was detected in a total of 3/40 vaccinated animals. Among the 20 animals that were vaccinated with rZH501-
\Delta NSs:GFP-\Delta NSm, two animals developed a peak postchallenge viremia on day 3 of 1.1 \times 10^2 and 7.0 \times 10^1 PFU eq/ml of whole blood, respectively (Table 1). Of the 20 animals vaccinated with rZH501-
\Delta NSs:GFP-\Delta NSm, two animals developed a peak postchallenge viremia on day 4 of 1.5 \times 10^5 PFU eq/ml of whole blood (Table 1). In all three animals, the detectable viremia was transient and was resolved by day 7 postchallenge (data not shown). No other vaccinated animals (37 total) had a detectable RVF viremia at any time point assayed postchallenge. In sharp contrast, the five sham-inoculated animals all suffered severe to lethal clinical illness, with 3/5 animals succumbing to infection by day 3 postchallenge, with peak viremia titers of 1.9 \times 10^7, 2.7 \times 10^7, and 3.1 \times 10^7 PFU eq/ml, respectively (Table 1 and data not shown.) Two sham-inoculated animals did not succumb to infection but did develop severe clinical illness (ruffled fur, hunched back, lethargy) with a peak viremia postchallenge of 2.7 \times 10^6 and 5.8 \times 10^5 PFU eq/ml whole blood, respectively (Table 1 and data not shown). As hypothesized, both candidate vaccines (rZH501-\Delta NSs:GFP and rZH501-\Delta NSs:GFP-\Delta NSm) significantly reduced postchallenge viremia (P < 0.0001) regardless of the dose. Additionally, Kaplan-Meier survivor analysis (log-rank and Wilcoxon tests) of survival postchallenge revealed a significant protective efficacy effect with both vaccine candidates, regardless of the dose, compared with that of the sham-inoculated controls (P < 0.001).

**Differentiation of wild-type-infected animals from vaccinated animals based on anti-NP/anti-NSs serology.** Serum obtained from all vaccinated and sham-vaccinated animals at day 26 postvaccination was tested for the presence of anti-NP- and anti-NSs-specific antibodies utilizing Vero E6 cells transfected with plasmids expressing either NP or NSs protein. As a positive control, the serum obtained from the two sham-vaccinated animals that survived infection and the six additional control rat sera (taken from animals inoculated with a sublethal dose of RVF virus for validation purposes) were utilized. As expected, control animals that survived infection developed high levels of both anti-NP and anti-NSs antibodies, with strong immunostaining of both in the in vitro-expressed cytoplasmic NP and the filamentous intranuclear accumulations of NSs protein (Fig. 3A). In concordance with the anti-RVF virus total IgG data, all vaccinated animals demonstrated high anti-NP-specific antibody levels, and as predicted, no vaccinated animal, regardless of vaccine virus or dose, developed detectable anti-NSs specific antibody (Fig. 3B). As a further step toward demonstrating the ability to differentiate naturally infected animals from those vaccinated with these sorts of prototype vaccines, naturally infected livestock were also shown to produce anti-NSs antibodies similar to those observed with the wild-type virus-infected rats (Fig. 3D).

**DISCUSSION**

Here we report the successful generation of multiple recombinant eGFP-tagged RVF viruses containing either full-length complete virus genomes or deletions of the NSs gene alone or the NSs/NSm genes in combination. Utilizing the technique of reverse genetics, we were able to rapidly create rRVF viruses containing an in vitro reporter moiety that is potentially useful as a live-virus research tool in the high-throughput screening of antiviral compounds and in the creation of recombinant virus containing precisely defined deletions of major virus virulence factors on multiple genome segments. This work constitutes the first report of in vitro and in vivo testing of rationally designed marker vaccine candidates for RVF virus.

Efforts to prevent RVF virus infection via vaccination began shortly after the first isolation of the virus in 1931 (19). These earliest vaccines (31) and several that followed, including the currently available TSI-GSD-200 preparation, relied on formalin inactivation of live wild-type virus (43, 45). While these inactivated vaccines are capable of eliciting protective immune responses among livestock and humans, they typically require a series of two to three initial inoculations, followed by regular booster vaccinations to achieve and maintain protection (43, 54). Unfortunately, multiple dosing and annual vaccination regimens are logistically difficult to implement and expensive to maintain and, thus, are of limited practical value in resource-poor settings, especially in regard to the control of RVF virus infection in livestock in enzootic settings. In addition, there have been problems in the past with quality control and “inactivated” vaccines causing disease problems.

In an effort to eliminate the necessity of booster inoculations, several live-attenuated vaccine candidates were developed for RVF virus, with some, such as the Smithburn neurotropic strain, being employed in Africa. These vaccine candidates have relied upon the random introduction of attenuating mutations via serial passage in suckling mouse brain or tissue culture, on in vitro passage in the presence of chemical mutagens such as 5-flourouracil, or as naturally occurring virus isolates (i.e., the Smithburn neurotropic or the Kenyan-IB8 strains, MP-12, or the clone 13 isolate, respectively) (11, 16, 39, 48, 52). Due to the technical limitations of these techniques and the lack of a complete genome sequence for many of the historically derived RVF virus vaccines, the exact underlying molecular mechanisms of attenuation for many of these live-attenuated RVF virus vaccines used throughout Africa or only in experimental settings is either unknown (Smithburn neurotropic strain or Kenyan-IB8) or reliant on the combinatorial effects of multiple nucleotide or amino acid substitutions (e.g., MP-12) (49, 55). Unfortunately, experimental and field experience with existing live-attenuated RVF virus vaccines demonstrated that in certain instances these vaccines retain the ability to cause teratogenic effects, abortion, and neural pathology in livestock or animal models make their widespread use potentially problematic, especially in nonendemic areas or during interepizootic/epidemic periods (26, 36–38).

While these vaccines are useful in many situations, several
distinct disadvantages exist among live-attenuated RNA virus vaccines prepared by these traditional techniques. Live-attenuated vaccines that rely on single or multiple nucleotide substitutions rather than whole-gene deletion are at increased risk for reversion to virulent phenotypes due to the inherently high rate of viral RNA polymerase errors. The loss of attenuation via this mechanism among livestock and human live vaccines is well documented (5, 12, 23, 25, 44). The potential for a similar reversion event among RVF virus live vaccines dependent on attenuating nucleotide mutation was illustrated by recent genomic analyses of RVF virus that revealed an overall molecular evolution rate ($\approx 2.5 \times 10^{-4}$ nucleotide substitutions/site/year) similar to that of other single-stranded RNA viruses (9). Due to error-prone polymerases, live-attenuated RNA virus vaccines prepared by the multiple serial passage techniques involved in virus attenuation inherently consist of a complex mixture of genomic microvariants. In contrast, the origin of reverse genetics-derived virus vaccine candidates is advantageous in that vaccine stocks can be generated directly or following limited amplification steps from precisely defined DNA plasmids. This approach should allow for the simple production of virus vaccines following good manufacturing processes with higher levels of genetic homogeneity.

Another significant drawback of all previously available live-attenuated RVF virus vaccines is that they do not allow for the DIVA. This ability is critical if a vaccination strategy is to be used to augment efforts to contain an accidental or intentional release of wild-type RVF virus in previously unaffected areas (24). As a high-consequence pathogen, RVF virus has been classified as a category A select agent as defined by the U.S.
Department of Health and Human Services and the Department of Agriculture (USDA) and is listed as a high-consequence agent with potential for international spread (list A) by the Office International des Epizooties (OIE) (29) of the World Organization for Animal Health (WOAH), thus greatly increasing the consequences for international livestock trade following the introduction of RVF virus into previously unaffected countries or epizootics in enzootic areas (57). Currently, OIE regulations require surveillance and the absence of RVF virus activity for 2 years following an outbreak before the resumption of disease-free status and the subsequent easing of import/export trade restrictions (27). The use of any current commercially available livestock vaccines does not permit the differentiation of vaccinated from naturally infected livestock, thus contraindicating the use of prophylactic vaccination in countries wishing to retain disease-free status or in those with ongoing/endemic RVF virus activity.

In order to potentially address these issues, we generated via reverse genetics infectious virus containing either complete deletions of major virus virulence factors, the NSs (rZH501-ΔNSs-GFP) or NSs and NSm (rZH501-ΔNSs:GFP-ΔNSm) genes, to confer attenuated phenotypes in vivo and to allow for the serologic differentiation of naturally infected and vaccinated animals by the presence/absence of anti-RVF virus NP/anti-RVF virus NSs antibodies. During in vivo testing, these rRVF virus vaccine candidates were found to be highly immunogenic and efficacious in the prevention of severe RVF virus disease and lethality (Fig. 2 and Table 1). In an initial pilot study, animals developed high end-point titers (≥1:400) of total anti-RVF virus IgG by day 21 postvaccination that were significantly higher than those of the sham-inoculated controls (P < 0.05; Table 1). Importantly, at no observed time point postvaccination did any animal develop disease symptoms or vaccine-induced viremia (Table 1).

Additional testing with a larger follow-up study confirmed these results, with the majority of animals generating robust total anti-RVF virus IgG responses, with typical titers of ≥1:6,400 by day 26 postvaccination, again with no detectable vaccine-induced viremia. The slightly higher, but not statistically significant, mean total IgG SumOP values, end-point titers, and PRNT50 values found among animals vaccinated with the rRVF-ΔNSs:GFP virus than among the animals vaccinated with the double-deletion ΔNSs/ΔNSm virus was likely due to the enhanced attenuation and reduced in vivo virus replication conferred by the disruption of both the NSs and the NSm RVF virus virulence factors. However, regardless of this slight reduction in total anti-RVF virus IgG, the immunologic response generated in the ΔNSs/ΔNSm virus-vaccinated animals was significantly higher than that of controls (P value of <0.05) and was sufficient to confer complete protection from both clinical illness and lethality in 100% of the vaccinated animals given a known lethal challenge dose of wild-type RVF virus (Table 1).

Direct comparisons of the level of protective immunity (PRNT50 or total IgG) titers with those of previous studies utilizing other RVF virus vaccines are difficult due to the vaccine used and the species level differences in immunity. However, earlier work utilizing the three-dose regimen (days 0, 7, and 28) of inactivated TSI-GSD-200 vaccine with the Wistar-Furth rat model demonstrated protective efficacy against virulent virus challenge at a PRNT50 of >1:40 (2). Later, a large retrospective analysis of human volunteers (n = 598) receiving the same recommended three-dose regimen of this inactivated vaccine found that subjects developed a mean PRNT50 of 1:237 (43). Additionally, a large study of the pathogenesis and neurovirulence of the live-attenuated MP-12 vaccine in rhesus macaques demonstrated PRNT50 values among vaccinated animals of ≥1:640 (38). In the present study, the mean PRNT50 among groups was found to range from 1:640 to 1:7,040, suggesting that the level of neutralizing antibody was roughly equivalent to that demonstrated in earlier RVF virus vaccine studies of animal model systems or among human volunteers.

These results suggest that the enhanced safety, the attenuation, and the reduced possibility of reversion to full virulence via either RVF virus polymerase nucleotide substitution or gene segment reassortment with field strains afforded by the double-genetic deletions of the entire RVF virus NSs and NSm genes does not significantly decrease overall vaccine efficacy. The high level of protective immunity induced by a single dose of either of these vaccine candidates was striking, with 37 of 40 total vaccinated animals developing a potentially sterilizing immunity as determined by the lack of any detectable postchallenge viremia (Table 1).

The challenge virus dose level utilized in these studies (1.0 × 10^3 PFU) of the virulent strain ZH501 was derived from previous studies (3, 4, 7, 42), indicating that this dose was equivalent to approximately 500-fold that of the 50% lethal dose in 6- to 8-week-old Wistar-Furth rats. The finding that 2/5 nonvaccinated animals survived this challenge dose was unexpected. While 3/5 animals rapidly developed lethal RVF virus disease, characterized by fulminant hepatic necrosis and a high viremia of approximately 1.0 × 10^7 PFU eq/ml, the remaining two control animals suffered severe clinical illness but developed lower peak viremia levels of 2.7 × 10^4 and 5.8 × 10^5 PFU eq/ml, respectively, which resolved by day 7 postchallenge. The lack of uniform lethality was likely due to the reduced susceptibility of the older animals at the time of virus challenge (10 to 12 weeks). While they are rare, animals surviving the 1.0 × 10^3 PFU dose have been reported previously (42). In the present study, regardless of the final lethality outcome, there was complete absence of disease, a significant reduction in postchallenge viremia between vaccinated and control animals (P < 0.001), and significant increases in survival (P < 0.001), reflecting the protective efficacy of these vaccine candidates.

As expected, no animal immunized with either of the vaccine candidates was found to have detectable anti-NSs antibodies. This finding, when coupled with the high level of anti-NSs antibody in survivor control animals, demonstrated that the DIVA may be possible among animals immunized with these candidate vaccines, based on the presence/absence of the anti-NSs antibody (Fig. 3A, B, and C). The experimental data presented here and the presence of anti-NSs antibody in the serum of naturally infected convalescent livestock obtained during the outbreak in Saudi Arabia in 2000 (Fig. 3D) and from human sera (data not shown) suggest that the use of these vaccine candidates combined with the further development of rapid enzyme-linked immunosorbent assays or solid matrix-based differential detection assays for anti-NP/anti-NSs antibodies may provide a robust DIVA field screening tool. These results taken together are very promising and strongly support the further development of rRVF virus vaccines containing
deletions of the NSs and NSm genes, such as rZH501-NSs:

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3. Training Program (VSTP) of the University of California, Davis, School
Science and Education (ORISE) Oak Ridge, TN, and by the Students

4. thanks N. James Machachlan for his steadfast support and encourage-

5. in genome sequencing during the completion of these studies. B.B.

6. health threat into previously unaffected areas.

7.ing natural or intentional introduction of this significant public

8. risk occupational settings or for recognized risk groups follow-

9. provide effective prophylactic protection for humans in high-

10. national regulatory approval process. An important final con-

11. fined attenuating deletions and the use of cDNA technology

12. potentially eliminating the need for resource-intensive fol-

13. low-up booster inoculations. Additionally, the precisely de-

14. protected from viru-

15. lent virus challenge, these safety features are critical for wide-

16. spread acceptance and adoption of any RVF virus vaccine among

17. commercial livestock farmers and pastoralists in affected areas

18. during interepizootic/epidemic time periods.

19. From an important practical standpoint, these vaccine can-

20. didates routinely grew to high titers in tissue culture and pro-

21. vided protective immunity following a single injection, thus

22. likely reducing the overall economic cost of production and poten-

23. tially eliminating the need for resource-intensive follow-

24. up booster inoculations. Additionally, the precisely de-

25. defined attenuating deletions and the use of cDNA technology

26. eliminates the potential risk of reversion to, or contamination

27. from, virulent wild-type virus inherent in serially passaged or

28. inactivated vaccine preparations and may ease the federal/

29. national regulatory approval process. An important final con-

30. sideration is that while targeted toward veterinary medical use

31. and thus indirectly the prevention of human RVF virus dis-

32. ease, the candidate vaccines presented here could potentially

33. provide effective prophylactic protection for humans in high-

34. risk occupational settings or for recognized risk groups follow-

35. ing natural or intentional introduction of this significant public

36. health threat into previously unaffected areas.

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