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A glycoprotein subunit vaccine elicits a strong Rift Valley fever virus neutralizing antibody response in sheep

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Abstract

Rift Valley fever virus (RVFV), a member of the *Bunyaviridae* family, is a mosquito-borne zoonotic pathogen that causes serious morbidity and mortality in livestock and humans. The recent spread of the virus beyond its traditional endemic boundaries in Africa to the Arabian Peninsula coupled with the presence of susceptible vectors in non-endemic countries has created increased interest in RVF vaccines. Subunit vaccines composed of specific virus proteins expressed in eukaryotic or prokaryotic expression systems are shown to elicit neutralizing antibodies in susceptible hosts. RVFV structural proteins, N-terminus glycoprotein (Gn) and C-terminus glycoprotein (Gc), were expressed using a recombinant baculovirus expression system. The recombinant proteins were reconstituted as GnGc subunit vaccine formulation and evaluated for immunogenicity in a target species, sheep. Six sheep were each immunized with a primary dose of 50 µg of each vaccine immunogen adjuvanted with montanide ISA25, and at day 21 post-vaccination, each animal received a second dose of the same vaccine. The vaccine induced strong antibody response in all animals as determined by indirect enzyme-linked immunosorbent assay (ELISA). Plaque reduction neutralization test ($PRNT_{80}$) showed the primary dose of the vaccine was sufficient to elicit potentially protective virus neutralizing antibody titers ranging from 40 to 160, and the second vaccine dose boosted the titer to more than 1,280. Further, all animals tested positive for neutralizing antibodies at day 328 pv. ELISA analysis using the recombinant nucleocapsid protein as a negative marker antigen indicated that the vaccine candidate is DIVA (differentiating infected from vaccinated animals) compatible, and represents a promising vaccine platform for RVFV infection in susceptible species.

Key words: Rift Valley fever virus, glycoproteins, subunit vaccine, neutralizing antibodies, sheep

Introduction

Rift Valley fever virus (RVFV) is a mosquito-borne zoonotic pathogen that causes high morbidity and mortality in both livestock and humans. The virus has caused outbreaks in ruminants and humans in Africa and the Arabian Peninsula (Flick and Bouloy 2005) and represents a threat to the wellbeing and livelihood of resource-poor farmers in much of Africa (Smith, et al. 2013). It is classified as a select agent and risk group-3 pathogen by the Centers for Disease Control and Prevention (CDC) and the United States Department of Agriculture (USDA). In ruminant livestock, Rift Valley fever (RVF) is characterized by high mortality in young animals, notably in lambs, fetal malformations and widespread abortion storms; sheep are the most susceptible, with neonatal mortalities approaching 100% (Swanepoel and Coetzer 1994). Human infections are often characterized by benign fever but in small proportion of individuals could lead to more serious complications such as retinitis, encephalitis, neurological disorders, hepatic necrosis, or fatal hemorrhagic fever (Bird, et al. 2009). Although human fatal hemorrhagic cases have been historically estimated at 2% in infected individuals, case fatalities of up to 20% have been reported (Bird, et al. 2009) including the recent outbreak in Mauritania (Heald 2012). The reason or factors contributing to this increase in fatalities are unknown.

RVFV belongs to the genus *Phlebovirus* within the family *Bunyaviridae*, which includes over 350 named isolates (Walter and Barr 2011). It has a tripartite single-stranded RNA genome of negative polarity consisting of small (S), medium (M) and large (L) RNA segments. The M segment encodes the two structural glycoproteins, Gn/Gc, the 78-kDa protein and the non-structural protein, NSm, whereas the S segment encodes the nucleocapsid protein (N) protein and the non-structural protein, NSs. The L-segment encodes the RNA-dependent RNA polymerase (Elliott 1996). The N and L proteins are required for viral RNA synthesis; and the NSs protein, the major virulence factor, has been shown to inhibit host transcriptional immune response (Bouloy, et al. 2001) through generalized transcription downregulation including

repression of IFN-β (Billecocq, et al. 2004,Le May, et al. 2004,Le May, et al. 2008) and degradation of protein kinase R (Habjan, et al. 2009,Ikegami, et al. 2009). The NSm protein functions to suppress virus-induced apoptosis (Won, et al. 2007) and there is evidence that it plays a functional role in the vector competence of mosquitos for RVFV at the level of the midgut barrier (Kading, et al. 2014). The glycoproteins, Gn and Gc, are surface proteins that play a role in virus attachment to initiate infection and have been shown to carry epitopes that elicit the production of neutralizing antibodies, a correlate of protective immunity (Besselaar and Blackburn 1992,Besselaar and Blackburn 1994,Besselaar, et al. 1991).

There are currently no RVFV vaccines fully approved for commercial use outside its endemic area in Africa and the Arabian Peninsula. Given the potential for viral spread elsewhere including the mainland US, there is an urgent need for a safe and efficacious vaccine. Attributes essential for a vaccine for use in non-endemic areas include safety and the ability to generate a rapid (with primary vaccination) protective immune response in a susceptible host. In endemic regions, RVFV in livestock has been controlled traditionally by using live-attenuated Smithburn strain or inactivated whole virus (Grobbelaar, et al. 2011). The Smithburn vaccine is highly immunogenic but is teratogenic in pregnant sheep and cattle (Botros, et al. 2006,Coetzer and Barnard 1977). The formalin inactivated whole-virus vaccines are safe but less immunogenic (Lubroth, et al. 2007). Clone 13, another live attenuated natural mutant, is now in commercial use in South Africa for the control of the disease in livestock (Muller, et al. 1995,Dungu, et al. 2010). MP12, a chemically attenuated virus derived from ZH548, an Egyptian wild-type isolate (Caplen, et al. 1985,Vialat, et al. 1997) is being evaluated as a potential vaccine for human and veterinary use. The immunogenicity and pathogenicity of these latter two candidate vaccines have been evaluated in various animal species (Muller, et al. 1995,Morrill, et al. 1997) and although both vaccine candidates showed promising results, the MP12 was reported to induce fetal malformations during the first trimester (Hunter, et al. 2002); however a recent study

reported the absence of fetal malformation in pregnant ewes inoculated with the virus (Morrill, et al. 2013). Strategies to develop RVFV vaccines include subunit (Mandell, et al. 2010,Naslund, et al. 2009,Schmaljohn, et al. 1989), DNA (Lagerqvist, et al. 2009,Spik, et al. 2006), virus-like particles (VLPs) (Naslund, et al. 2009,de Boer, et al. 2010,Kortekaas, et al. 2012), virus replicon particles (Dodd, et al. 2012,Kortekaas, et al. 2011,Oreshkova, et al. 2013), virus-vectored (Heise, et al. 2009,Wallace, et al. 2006), modified live vaccines, developed from recombinant viruses engineered using reverse genetics (Bird, et al. 2008,Bird, et al. 2011,Billecocq, et al. 2008,Habjan, et al. 2008,Ikegami, et al. 2006), live attenuated (Muller, et al. 1995,Dungu, et al. 2010,Caplen, et al. 1985,Morrill, et al. 2013,Smithburn 1949,Pittman 2012) and inactivated whole virus vaccines (Pittman, et al. 2000). Although subunit vaccines for RVFV are generally considered safe and recently some progress has been made in their development, evaluation of immunogenicity and/or efficacy in a target species, sheep, has been performed for a few candidates (Kortekaas, et al. 2012,Oreshkova, et al. 2013). On the other hand, production of live-vaccines requires high level of biosafety; and their use is associated with potential risk to personnel and reversion to virulence in vaccinated hosts. Therefore, the general availability of a safe, efficacious vaccine with DIVA compatibility will be extremely valuable to endemic and non-endemic countries outside Africa.

In this study, we describe the expression of the RVFV structural glycoproteins, Gn and Gc, and assess their immunogenicity in a natural target species, sheep. The results demonstrate the induction of a strong virus neutralizing antibody response with potentially protective titers induced within two weeks after the primary vaccination without any noticeable adverse reactions. Taken together, the findings suggest that a baculovirus-expressed RVFV glycoprotein subunit vaccine could be a potential viable candidate for development of an effective vaccine against RVFV.

MATERIALS AND METHODS

Cell cultures

The African green monkey cells, Vero E6 (ATCC, Manassas, VA), were maintained in Eagle's minimal essential medium (Corning Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum, L-glutamine and penicillin-streptomycin (Invitrogen-Life Technologies, Carlsbad, CA). The cultures were maintained at 37^{o}C in humidified 5% CO_2 atmosphere. The *Spodoptera frugiperda* (Sf9) cells (Invitrogen-Life Technologies) were maintained in SFM 900 II medium supplemented with 10% fetal bovine serum and penicillin-streptomycin (Invitrogen-Life Technologies). These cells were maintained at 27^{o}C.

Construction of recombinant bacmid

The cloning and construction of recombinant bacmid was carried out as described previously (Faburay, et al. 2013). Briefly, the coding sequences of the RVFV structural proteins, Gn and Gc, were retrieved from the RVFV ZH548 isolate sequences (GenBank Accession No. DQ380151) and the sequences were synthesized (Genewiz, San Diego, CA) with molecular modifications. For Gn, the transmembrane domain and cytoplasmic tail were deleted from the coding sequence, and designated as Gn ectodomain (Gne) (Figure 1a). To ensure translocation of both Gne and Gc into the endoplasmic reticulum (ER) and processed through the cellular glycosylation pathway, a specific 54-nucleotide signal peptide (SP) sequence was identified in the M segment of the RVFV genome as described previously (Faburay, et al. 2013); sequences are available on request. This SP sequence was fused to the 5' end of the Gne and Gc sequences (Figure 1b). The signal peptide contained a cleavage site and cleavage is catalyzed by signal peptidases located in the lumen of the ER, a site for protein translation and posttranslational modification. The nucleoprotein (N) nucleotide sequence was amplified by

PCR from pET30 Ek/LIC recombinant plasmid, a gift from Dr. Friedeman Weber, University of Marburg, Germany, which contained the entire coding region of the S segment of the RVFV strain ZH548. The RVFV-specific sequences were cloned into pFastBac/CT-TOPO vector (Invitrogen-Life Technologies) in-frame with C-terminal polyhistidine-tagged protein resulting in donor plasmids, pRF-Gne, pRF-Gc and pRF-N. The individual donor plasmids were transformed into MAX Efficiency DH10Bac competent *E. coli* to generate recombinant bacmids. Recombinant bacmids were purified and used for transfection to express the respective proteins.

Recombinant baculovirus expression and purification of RVFV proteins

Recombinant baculovirus expression of the respective RVFV proteins was carried out as described previously (Faburay, et al. 2013). Briefly, purified recombinant bacmids carrying the respective coding sequences (Gne, Gc and N) of RVFV were transfected, using CellfectinII reagent (Invitrogen-Life Technologies), into Sf9 cells (Invitrogen-Life Technologies) grown in Sf-900 II SFM medium (Invitrogen-Life Technologies) supplemented with 10% fetal bovine serum and 100U/ml-100 µg/ml penicillin-streptomycin. Protein expression was carried out using P2 or higher passage recombinant baculovirus stock (>10^7 pfu/ml). Western blot analysis of the lysate was performed to confirm specific protein expression as described below. The proteins were expressed with a C-terminal 6xHis-tag and purification using Ni-NTA superflow resin (Novagen, Rockland, MA) was performed according to the protocol described previously (Faburay, et al. 2013). The purified proteins were stained with Coomassie Blue, and protein concentrations were determined using the bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL) at an absorbance of 562 nm, using bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, Mo) as the protein standard. Aliquots were stored at – 80^{o}C until used.

Expression and purification of RVFV N protein

Recombinant *E. coli* expression of RVFV N protein was carried out using an expression construct kindly provided by Dr. Friedeman Weber, University of Marburg, Germany. The *E. coli* cells Bl21(DE3)pRARE harboring the expression vector pCDNA3.1 were grown on Luria Bertani (LB) agar plates containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. A single clone was picked and inoculated into 10 ml LB media for overnight growth. Ten milliliters of the overnight culture was then inoculated into 1 liter of LB medium, and expression was induced with the addition of 0.4 mM IPTG when OD_{600} reached 0.6. The culture was grown overnight at 15°C. Cells were harvested by centrifugation at 4,500 x g for 15 min at 4°C, resuspended in 1 ml lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl) and lysed by three freeze-thaw cycles and then sonicated three times for 5 sec each (Sonic Dismembrator, Model 100, Fischer Scientific, Inc.) . The lysate was fractionated by centrifugation for 30 min at 10,000 x g. The supernatant was clarified through 0.45 µm filter and then loaded onto a 5 ml HisTrap HP column (GE HealthCare) in conjunction with an AKTA Xpress purification system at flow rate of 1 ml /min. The columns were washed with Buffer A (50 mM Tris, 300 mM NaCl, pH=8.0) over 10 column volumes. Non-specifically bound proteins were removed by washing with 5 column volumes of 10% Buffer B (50 mM Tris, 300 mM NaCl, pH=8.0, 500 mM imidazole). Protein was eluted from the column with a gradient of 10-100% Buffer B over 10 column volumes. The elution fractions, which contained the purified N protein were pooled and stored at -80^0C after addition of equal volume of 100% glycerol.

Detection of recombinant protein expression and analysis of immunoreactivity

The procedure for detection of recombinant protein expression by Western blot has been described previously (Faburay, et al. 2013). Expression of recombinant proteins, N and Gne, was further confirmed using primary antibodies, mouse anti-N (R3-ID8) and mouse anti-Gn monoclonal antibody, 4D4, (a gift from Dr. Connie Schmaljohn, United States Army Medical

Research Institute for Infectious Diseases), respectively at a dilution of 1:2,000. To probe immunoreactivity of antisera obtained from sheep vaccinated with recombinant RVFV Gne and Gc glycoprotein subunit vaccine, the blots were incubated with individual sheep sera at 1:100 dilution for 1hr at room temperature. After washing, the membrane was incubated for 1 hr at room temperature with Protein G-HRP (Abcam, Cambridge, MA) diluted 1: 25,000. After the final washing steps, specific reactivity was detected using AEC (3-Amino-9-ethyl-carbazole) peroxidase substrate (Sigma-Aldrich, St. Louis, MO) or ECL enhanced chemiluminescent detection system.

Preparation of the vaccine, animal immunization and MP12 virus antiserum

The purified glycoproteins were formulated in montanide ISA25 water-in-oil adjuvant (Seppic, France) to obtain a concentration of 50 µg of each immunogen per vaccine dose according to the manufacturer's instruction. Six adult sheep (#9, #36, #163, #169, #170, #179), Rambouillet breed, were each immunized subcutaneously with a primary dose of 50 µg of purified Gne and 50 µg of purified Gc. At day 21 post-primary vaccination, hereafter referred to simply as postvaccination (pv), each sheep was given a booster with the same amount of vaccine (a second dose). At different time points after vaccination, the injection sites were inspected for possible adverse reaction. Blood samples were collected from the jugular vein of each sheep for the separation of sera prior to vaccination (day 0), in order to establish baseline pre-vaccination immune response status. Thereafter, serum samples were collected from each sheep weekly on days 7, 14, 21, 28, 35, 42 and 49 pv. All sera were stored at -80^{o}C until used. The RVFV MP12 control antiserum was obtained from a sheep at day 28 postinfection in a previous experiment conducted at the Kansas State University Biosecurity Research Institute (BRI) (Faburay, et al. 2013). Animal experiments complied with institutional guidelines of South Dakota State University and were approved (Committee Approval No. 12-037A) by South Dakota State University Institutional Animal Care and Use Committee (IACUC).

Immunogen-specific antibody response

Specific antibody response in serum was measured by enzyme-linked immunosorbent assay (ELISA) using nickel column-purified baculovirus-expressed RVFV Gne, Gc and *E. coli*-expressed RVFV N proteins. The N protein was expressed in *E. coil* using an expression construct kindly provided by Friedeman Weber, University of Marburg. Germany, and was used as a negative diagnostic marker antigen to assess the DIVA compatibility of the recombinant subunit vaccine. To perform an ELISA, each well, in a 96-well plate (Nunc, Maxisorp) format, was coated overnight at 4^{o}C with approximately 100 ng of each purified recombinant protein in 100 µl of Dulbecco's coating buffer (pH 7.4) (Invitrogen-Life Technologies). The ELISA assays were performed as described previously (Faburay, et al. 2013). The cut-off point was determined for each sheep in each specific ELISA by the addition of two standard deviations to the corresponding mean OD value of the pre-vaccination serum. Mean OD values equal to or greater than the cut-off value were considered positive.

Anti-RVF virus $PRNT_{80}$

The stock of MP12 RVFV was diluted to 50 PFU in 250 µl of 1x MEM containing 4% bovine serum albumin (Sigma-Aldrich, St. Louis, MO). Separately, aliquots of serum from each vaccinated sheep were diluted as follows: 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, and 1:1280 in 1x MEM containing 2% bovine serum albumin and 1% penicillin streptomycin. Diluted serum (250 µl) was mixed with an equal volume of diluted MP12 virus and incubated at 37^{o}C for 1 hr. Thereafter, each mixture of serum plus RVFV was used to infect confluent monolayers of Vero E6 cells in 12-well plates. After 1 hr adsorption at 37^{o}C and 5% CO_2, the mixture was removed, and 1.5 ml of nutrient agarose overlay (1x MEM, 4% bovine serum albumin, and 0.9% SeaPlaque agar) was added to the monolayers. After 5 days incubation, the cells were fixed with 10% neutral buffered formalin for 3 hrs prior to removal of the agarose overlay. The

monolayer was stained with 0.5% crystal violet in PBS, and plaques were enumerated. The calculated $PRNT_{80}$ corresponded to the reciprocal titer of the highest serum dilution, which reduced the number of plaques by 80% or more relative to the virus control. As positive neutralizing serum control, a 1:40 dilution of day 28 serum obtained from a sheep previously immunized with RVFV GnGc VLP was used. The serum showed a $PRNT_{80}$ titer of 1,280 (data not shown). Studies in humans and non-human primates show that a $PRNT_{80}$ titer of ≥40 is protective against virulent RVFV challenge (Pittman, et al. 2000,Papin, et al. 2011); thus in this study a $PRNT_{80}$ titer of ≥40 was considered potentially protective.

Electron microscopy

Co-expression of RVFV glycoproteins, Gne and Gc, in cell culture has been shown to result in the formation of VLPs (Mandell, et al. 2010, de Boer, et al. 2010). Thus to rule out possibility of assembly of recombinant Gne and Gc upon mixing of the proteins into vaccine formulation, a transmission electron microscopy (TEM) was performed. Briefly, equal amounts of purified Gne and Gc were mixed together in a single tube and incubated for 30 min at room temperature. Alongside, aliquots of purified Gne and Gc were also made in separate tubes and incubated for 30 min as described above as controls. Subsequently, the proteins were nebulized on cupper Formar-carbon coated grids (Tedd Pella Inc., Redding, CA), dried at room temperature for 30 min and stained with phosphotungstic acid (PTA). Images were recorded at a calibrated magnification of 30,000x or 60,000x using an electron microscope (FEI Technai G2 Spirit biot, Hillsboro, Ore).

Statistical analysis

We used a t-test of independent samples with equal or unequal variances for statistical analysis. To determine the significance of observed differences in the optical density (OD) values of

prevaccination and postvaccination sera, a serum reactivity index (SRI) for each sheep, defined as the ratio of postvaccination serum OD value to the prevaccination serum OD value, was determined.

Results

Expression of RVFV N, Gn and Gc recombinant proteins

Recombinant baculovirus expression of the target proteins was analyzed by SDS-PAGE and Western blot. Using anti-hisHRP monoclonal antibody, expression of recombinant N, Gne and Gc glycoproteins of RVFV was detected. The expression of the recombinant proteins is a continuation of our previous work on the expression of RVFV structural and non-structural proteins, which confirmed the expression of Gne and Gc in glycosylated form. A ~31 kDa N protein, ~52 kDa Gne protein and a ~60 kDa Gc protein were detected (Faburay, et al. 2013). Expression of N protein and Gne glycoprotein was confirmed using mouse anti-N and anti-Gn monoclonal antibodies, respectively (Faburay, et al. 2013) . Reactivity of recombinant Gc was confirmed in Western blot using antisera obtained from RVFV-infected sheep (Faburay, et al. 2013). A Coomassie blue stain of purified Gne and Gc used as vaccine immunogens and in ELISA showed significant enrichment of the recombinant proteins with a substantial amount of the purified total protein (estimated at ≥ 80%) consisting of the target protein (Figure 1c). Additional Western blot analysis showed specific immunoreactivity of recombinant Gne and Gc with day 28 pv sheep sera, whereas recombinant N protein showed no specific reactivity (data not shown).

Vaccination and Immunogenicity of Gne and Gc glycoproteins

To examine vaccine-induced seroconversion and the kinetics of antibody response, sera collected from the vaccinated sheep at various time points pv (day 0, 7, 14, 21, 28, 35, 42 and

49), were tested in Gne and Gc-specific indirect ELISAs (Figure 2a and b). The vaccine immunogens, as well as antigens used in the ELISA assays, were prepared from purified proteins enriched for the recombinant glycoproteins (Figure 1c). Thus it was considered that the overwhelming antibody responses detected in the ELISAs were vaccine-induced and highly specific to Gne and Gc. Antibody reactivity with Gne antigen was detected in three of the sheep at day 7 pv (Figure 2a). At day 14 pv, all sheep seroconverted showing reactivity in the Gne and Gc-specific ELISAs, with Gne-specific antibodies showing comparatively stronger early-onset reactivity (Figure 2a and b). A second vaccine dose at day 21 pv significantly ($P < 0.05$) increased specific reactivity with both antigens at day 28 pv (Figure 2a and b). Serum reactivity index (SRI), a metric for vaccine-induced antibody response in vaccinated animals, showed an increase in OD values ranging from 4 to 9.6 fold, to the Gne antigen (Figure 2c) and 8 to 22.4 fold to the Gc antigen (Figure 2d). For both antigens, peak induction of antibody response was observed at day 28 pv (Figure 2a and b). Inspection of the injection sites revealed no adverse reaction (granuloma, skin ulceration, etc.) to vaccine administration.

DIVA compatibility of recombinant RVFV GnGc glycoprotein subunit vaccine

A DIVA (the ability to differentiate naturally infected from vaccinated animals) concept analysis by indirect ELISA was carried out using the Gne protein, as a positive diagnostic antigen, and N protein, as a negative marker, to detect specific antibodies in vaccinated sheep. Using sera from vaccinated sheep, an increase in immunoreactivity with Gne antigen was observed from day 7 pv to day 28 pv, which later plateaued until the end of the experiment (Figure 3a). In contrast, immunoreactivity with N-antigen was barely detectable, remaining at baseline levels throughout the experiment (Figure 3a). To confirm the specific immunoreactivity of the N protein as a marker antigen in ELISA, a day-28 MP12 postinfection antiserum (positive control) was tested alongside sera obtained from sheep vaccinated with the glycoprotein-based vaccine (Figure

3b). Reactivity with these sera remained at baseline negative levels at all the time-points, whereas the MP12 control serum showed strong reactivity as indicated by a high OD value ($P < 0.05$).

Recombinant RVFV glycoproteins elicit neutralizing antibody response

To examine vaccine-induced neutralizing antibody response, a plaque reduction neutralization assay was performed using an attenuated RVFV virus strain, MP12. A representative sample of a negative and positive $PRNT_{80}$ assay using prebled and day 28 pv sera (sheep #170), respectively, is shown (Figure 4a and b). In this study, a serum neutralizing antibody titer of 40 or greater is considered potentially protective (Pittman, et al. 2000,Papin, et al. 2011). Five of the six vaccinated sheep showed protective neutralizing titers at day 14 pv in response to the primary vaccination, with antibody titers ranging from 40 to 160, and one sheep, #9, showed protective neutralizing titer of 40 as early as day 7 pv (Table1). Protective levels of virus neutralizing titers were maintained in all the sheep until day 21 pv with three of the five sheep (#170, #179, #36) showing a titer increase. A second vaccine dose administered day 21 pv significantly boosted response in all six sheep above 1,280 titer, at day 28 pv (Figure 4c) (Table 1). Neutralizing antibody titers remained high in all sheep until day 49 pv (Figure 4c) and all animals were positive for RVFV neutralizing antibodies when tested at day 328 pv with titers ranging from 80 to 160 (Table 1).

Electron microscopy

The purpose of transmission electron microscopy (TEM) was to rule out the formation of VLPs following mixing of the recombinant glycoproteins, Gne and Gc, into vaccine formulation. Images revealed by TEM showed clumps of protein aggregates that showed no resemblance to VLPs (Figure 5).

Discussion

The impact of RVF outbreaks in Africa and the Arabian Peninsula, and the potential for viral spread to non-endemic areas, makes the development of safe and efficacious vaccines urgent. RVFV is a suitable candidate for a one-health focused approach to prevent both livestock and human disease through animal vaccinations (Oreshkova, et al. 2013). However, there are currently no fully licensed or commercial vaccines for human or livestock use outside endemic areas, despite numerous potential vaccine candidates. Essential attributes for a RVFV vaccine include safety and high immunogenicity, DIVA compatibility and the ability to induce a rapid onset of protective response with single vaccination, at most within two weeks of administration in susceptible host species. Herein, the immunogenicity of a recombinant baculovirus-expressed RVFV Gne and Gc glycoprotein-based vaccine candidate in a natural host species, the sheep, is reported. Gn and Gc are presented as glycosylated proteins on the surface of RVF virions and have been shown to carry epitopes that elicit neutralizing antibodies, the only established correlate of protective immunity against virus infection (Besselaar and Blackburn 1992,Besselaar, et al. 1991). They are also utilized by the virus for attachment to target cells (Schmaljohn and Nichol 2006). Thus the surface glycoproteins represent ideal targets for vaccine development; and we hypothesized that antibodies targeting epitopes on both structural glycoproteins in a subunit vaccine would generate a strong virus neutralizing effect. To produce vaccine immunogens, Gne and Gc expression constructs were designed to include a signal peptide at their N-terminus containing a signal peptidase cleavage site that ensures processing through translocation into the ER and the cellular glycosylation pathway and as well enhances protein expression (Faburay, et al. 2013). Subsequently, sheep were immunized with the purified baculovirus-expressed Gne and Gc proteins adjuvanted with montanide ISA25 resulting in induction of virus neutralizing antibody response, followed by a strong response after the booster dose, in all vaccinated animals. However, due to lack of approval at the time of this

study to perform challenge experiments with wild type RVFV, it was not possible to conduct further studies to test the efficacy of the vaccine. The vaccine induced potentially protective, (i.e. 1:40), virus neutralizing titers with single vaccination in five of the six animals within two weeks pv (Table 1). These results compared favorably with the outcome of recently reported vaccinations using vaccines based on RVFV glycoproteins, such as GnGc-VLPs and Gn-ectodomain (Mandell, et al. 2010,Naslund, et al. 2009,de Boer, et al. 2010,Kortekaas, et al. 2012,Oreshkova, et al. 2013), as well as a Newcastle Disease virus-vectored vaccine (NDFL-GnGc) (Kortekaas, et al. 2010,Kortekaas, et al. 2010) and virus replicon particles (Dodd, et al. 2012,Oreshkova, et al. 2013), some of which have also been reported to elicit neutralizing antibodies with single vaccination in sheep (Kortekaas, et al. 2012,Oreshkova, et al. 2013,Kortekaas, et al. 2010). Of significance, is that the GnGc-based recombinant protein vaccine candidate tested in this study induced potentially protective neutralizing antibody titers with single vaccination in 80% (5/6) of sheep within two weeks of vaccination and 100 % (6/6) sheep at three weeks pv (Table 1). The robust neutralizing antibody response elicited by the RVFV vaccine candidate could be attributed to the concurrent use of Gne and Gc proteins as vaccine immunogens. Gn is known to contain virus neutralizing epitopes, whereas inclusion of Gc in the vaccine is suggested to provide an additional target for neutralizing antibodies (Besselaar and Blackburn 1992,Besselaar, et al. 1991). Neutralizing antibody titers increased sharply in all animals following the booster and this high anamnestic response was maintained in all animals for more than three weeks, i.e., up to day 49 post vaccination. Remarkably, neutralizing antibody titers, ranging from 80 to 160, above the potentially protective threshold, was detected in all sheep at day 328 postvaccination (Table 1). This is against the backdrop that in the US, the withdrawal time for the adjuvant used in the current vaccine is 90 days. An early-onset vaccine-induced IgG antibody response to Gne occurred in half of the sheep within seven days pv followed by seroconversion in 100% of the animals at two weeks pv for both Gne and Gc (Figure 2a, b, c and d). It is noteworthy that high ELISA background was detected in

some sheep. Although this is speculated to be due to a host of unknown factors including the physiological status of individual animals, it nonetheless indicates the need for further development of the assay. Taken together, these results support the conclusion that the RVFV recombinant GnGc glycoprotein-based vaccine candidate is highly immunogenic, eliciting strong immune response in sheep, the natural and most susceptible species to RVFV.

Differentiating infected from vaccinated animals (DIVA) during RVF disease outbreaks is of fundamental epidemiological importance. Therefore, DIVA compatibility of a vaccine with accompanying diagnostic tests represents an important factor for consideration when designing vaccines especially for use in countries or regions non-endemic for RVFV. Using the RVFV glycoproteins and the nucleocapsid protein as diagnostic antigens, it was possible to distinguish vaccine-induced antibody responses (Figure 3a) from RVFV MP12 infection in sheep (Figure 3b). The increase in international trade in livestock coupled with the potential for RVFV outbreaks in non-endemic areas provides strong incentives for the development of DIVA vaccines. The absence of the nucleoprotein in the vaccine offers the possibility of developing a DIVA vaccine with a companion diagnostic assay using the recombinant N and Gne/Gc ELISA. The N protein represents a suitable diagnostic antigen as it is the most abundant viral protein and is highly immunogenic, inducing antibodies within the first days after infection (Faburay, et al. 2013,Swanepoel, et al. 1986,Paweska, et al. 2008). Furthermore, the recombinant GnGc glycoprotein subunit vaccine candidate elicited strong neutralizing and IgG antibody responses in the natural host, which can be easily detected by ELISA assays; analysis of the structural morphology of the vaccine immunogens by electron microscopy confirmed that the proteins upon reconstitution formed into clumps or aggregates which were very much distinct from VLPs (Figure 5). To date, RVFV VLP assembly has been reported to occur only by simultaneous production of Gn and Gc in both mammalian (Mandell, et al. 2010,Mandell, et al. 2010) and

insect cells (de Boer, et al. 2010,Liu, et al. 2008) and in all cases involved the co-expression of nonhistidine-tagged proteins. These results are anticipated, for unlike with naked viruses, where VLPs are re-assembled proteins, formation of an enveloped virus such as RVFV requires live cells.

RVFV represents a threat to human and animal health and there is no commercially available vaccine or effective antiviral therapeutic agent for human use. Efforts to produce live attenuated vaccines (Bouloy and Flick 2009) are tempered by safety concerns; however, such safety concerns are comparatively lower for recombinant protein-based vaccines. However, unlike live-attenuated vaccines, recombinant protein-based vaccines generally require booster to elicit long-term protective immune response. Therefore future research will be directed at further improving the duration and onset of protective neutralizing antibody responses. Additionally, the fact that RVFV has low genetic diversity and consists of a single serotype suggests that the recombinant Gne and Gc glycoprotein vaccine would likely confer protection against all strains of the virus. Further evaluation of the efficacy of the vaccine in livestock and non-human primates is the next step towards developing a safe and efficacious vaccine for livestock and human use.

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Author Disclosure Statement

The authors have declared that no competing financial interests exist.

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Tables

Table 1. Reciprocal $PRNT_{80}$ titers in sheep in response to vaccination with RVFV recombinant GnGc glycoprotein subunit vaccine

Sheep No.	Reciprocal $PRNT_{80}$ titers								
	Days: 0 prevac	7 pv	14 pv	21 pv	28 pv	35 pv	42 pv	49 pv	328 pv
169	0	0	40	40	1280>	1280>	1280>	1280	80
163	0	0	10	20	1280>	640	1280>	1280	80
170	0	0	40	80	1280>	1280>	1280>	1280>	80
179	0	0	40	80	1280>	1280>	1280>	1280	160
9	0	40	160	80	1280>	1280>	1280	1280	160
36	0	0	80	160	1280>	1280	1280	1280	80
Mean	0	nd	62	77	1280>	nd	1280>	1280>	107
Range	0	nd	10 – 160	20 – 160	1280>	640 – 1280>	1280 – 1280>	1280 – 1280>	80 - 160

prevac = prevaccination; pv = postvaccination; nd = not determinable

Figures

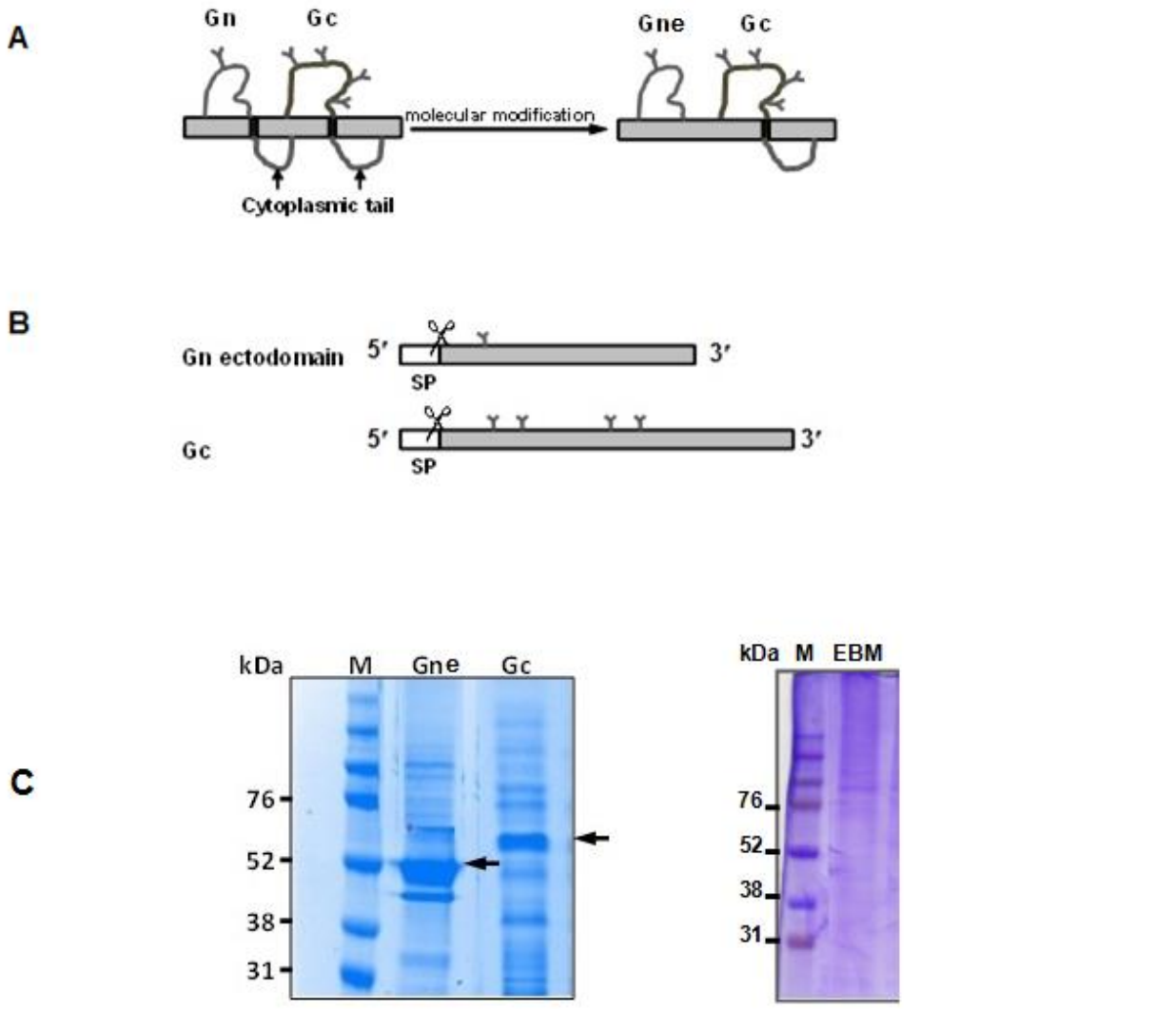

Figure 1. Creation of Gne and Gc recombinant constructs and recombinant protein expression. (A) shows diagrammatic representation of deletion of the transmembrane (TM) domain (black boxes) and the cytoplasmic tail of Gn. (B) shows diagrammatic representation of signal peptide (SP) with a signal peptidase cleavage site (scissors) identified in the M segment of RVFV genome is fused to the 5′ terminus of Gne and Gc. (ϒ) denotes putative glycosylation sites (Gerrard and Nichol 2007). (C) Coomassie blue stain of purified recombinant Gne, Gc and empty baculovirus-infected, mock purified cell lysate (EBM). M = molecular weight marker.

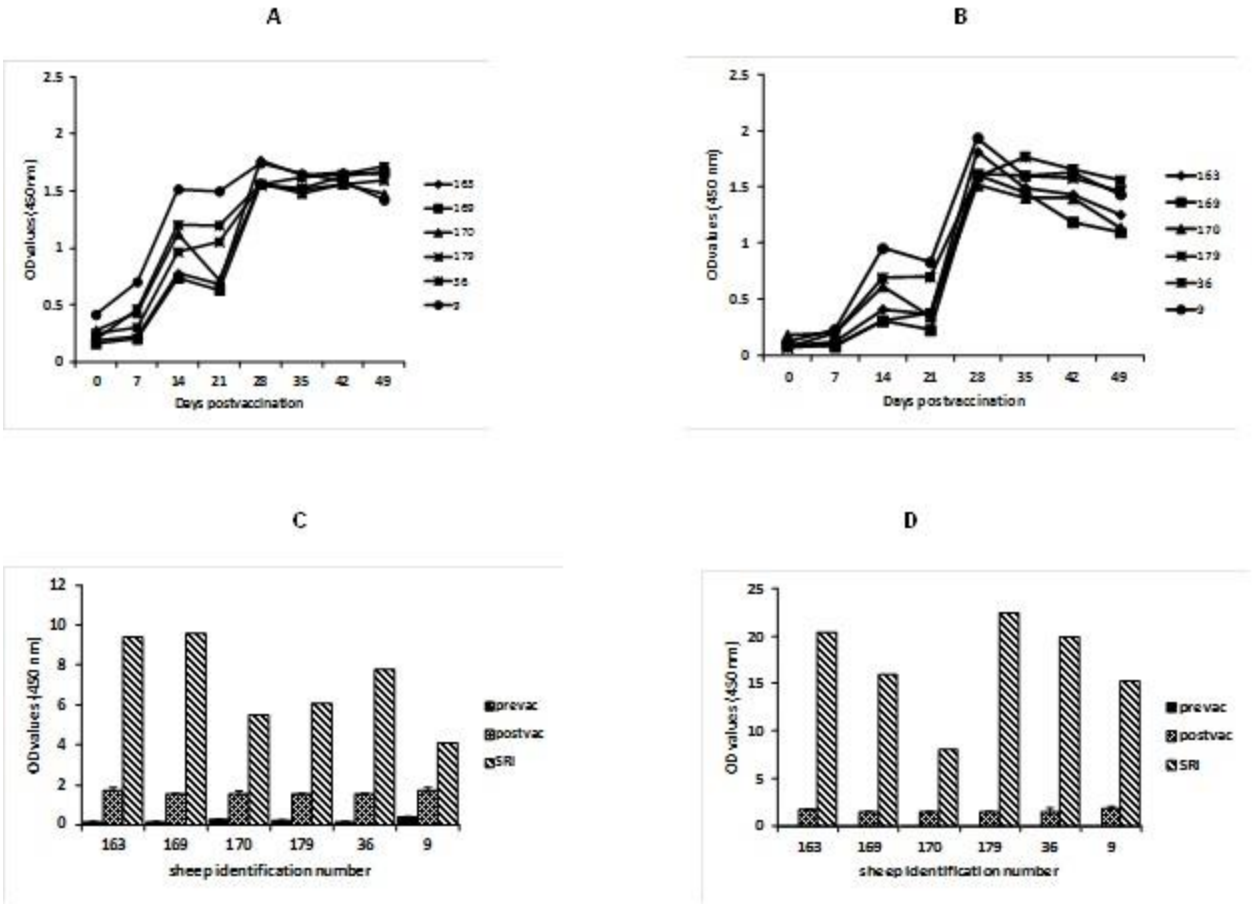

Figure 2. Analysis of vaccine-induced IgG host antibody response by antigen-specific indirect ELISAs, Gne-ELISA (A) and Gc-ELISA (B) depict time-dependent increase in specific antibody titer. Analysis of serum reactivity indices (SRI) using prebled sera against day 28 pv sera show significant increase in specific antibody titers ($P < 0.05$) demonstrated by high SRI values in both Gne-ELISA (C) and Gc-ELISA (D). Prevac = prevaccination serum; postvac = postvaccination serum; SRI = serum reactivity index. The cut-off value for individual sheep in Gne-ELISA: #163 = 0.354; #169 = 0.167; #170 = 0.507; #179 = 0.365; #36 = 0.252; #9 = 0.668. The cut-off value in Gc-ELISA: #163 = 0.215; #169 = 0.151; #170 = 0.309; #179 = 0.104; #36 = 0.7135; #9 = 0.259. A cut-off value was determined for each sheep as described in materials and methods.

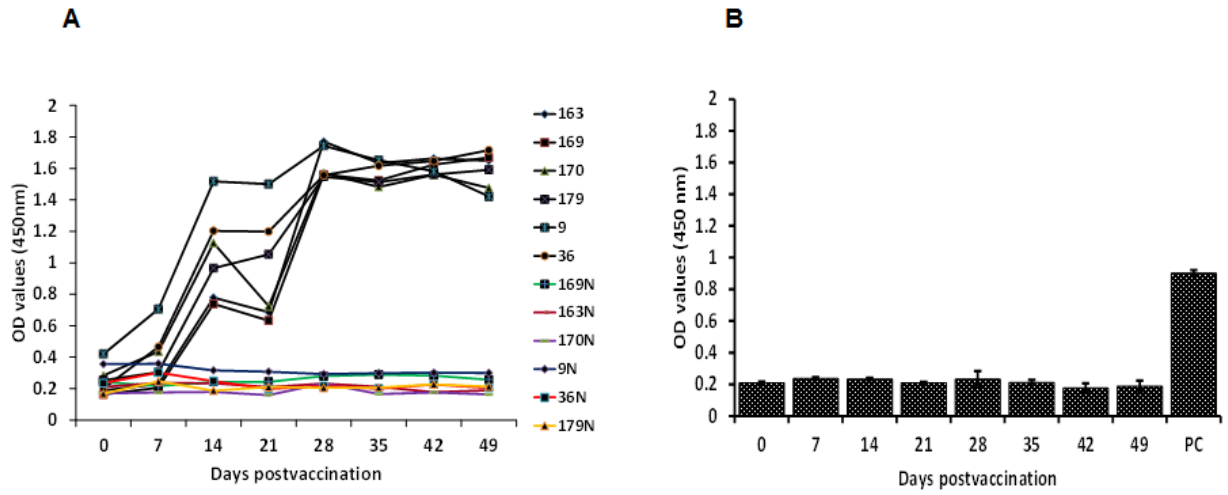

Figure 3. DiVA analysis of the subunit shows Indirect IgG ELISA of RVFV anti-Gn and anti-N antibody response in the vaccinated sheep. A) Reactivity of sera with Gne antigen indicates a time-dependent increase in antibody response, whereas in N-ELISA, reactivity remains at baseline prevaccination levels at all time points for all sera obtained from the vaccinated sheep. B) Comparison of the reactivity of sera obtained from sheep vaccinated with the glycoprotein-based subunit vaccine to sera obtained from RVFV MP12 infected sheep, the positive control serum (PC). The N antigen was positively reactive with only the positive control serum indicated by high mean OD value; day 0 to day 49 sera were obtained from sheep #169. The cut-off value for individual sheep in Gne-ELISA: #163 = 0.354; #169 = 0.167; #170 = 0.507; #179 = 0.365; #36 = 0.252; #9 = 0.668. The cut-off value in N-ELISA for individual sheep tested: #169N = 0.288; #163N = 0.237; #170N = 0.212; #9N = 0.407; #179N = 0.188; #36N = 0.239. N denotes recombinant N antigen used in ELISA. A cut-off value was determined for each sheep as described in materials and methods.

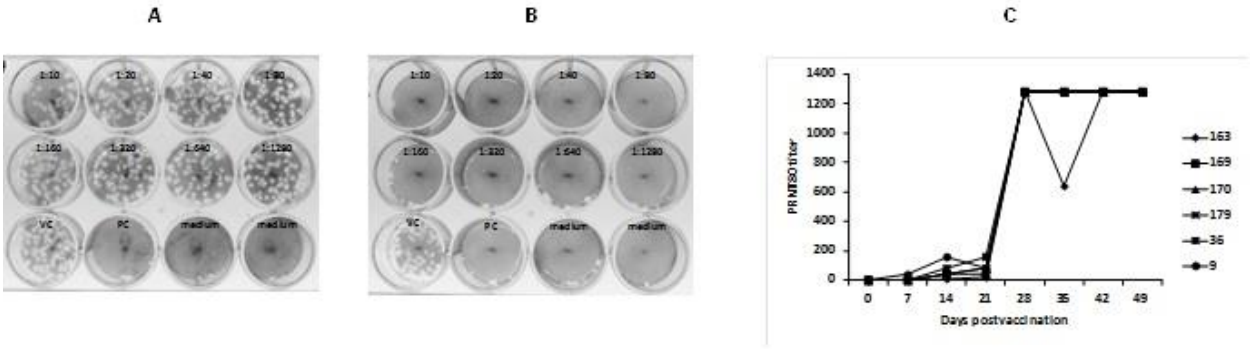

Figure 4. An illustration of plaque reduction neutralization test ($PRNT_{80}$) shows a negative (A) and a positive (B) RVFV neutralization test result. Protective levels of RVFV neutralizing antibody titers (≥40) are detectable in the animals within 2 weeks postvaccination. A marked increase in RVFV neutralizing antibody titer is detected in all animals following administration of the second vaccine dose (C). PC = positive control serum (1:40 dilution); VC = virus control (50 pfu).

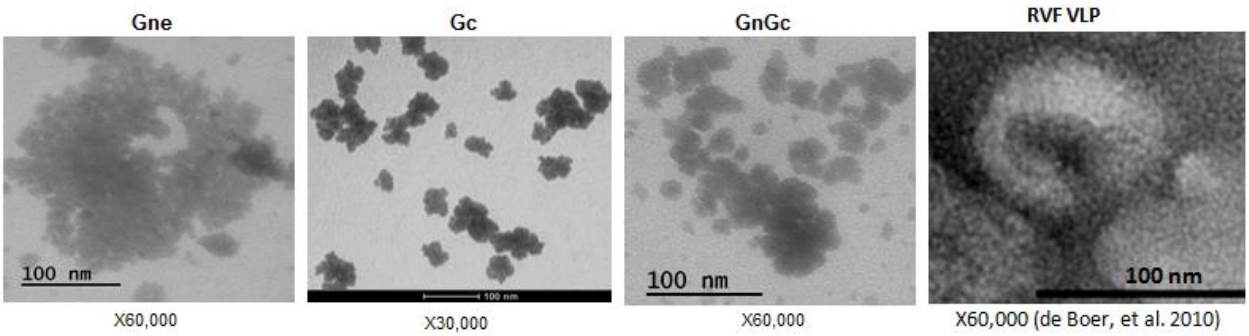

Figure 5. Electromicrograph of purified recombinant RVFV proteins used in the vaccine formulation. Recombinant Gne, Gc as well as mixed GnGc show clumps of protein aggregates that are structurally distinct from RVF VLPs, shown as control (de Boer, et al. 2010). Gne and Gc are mixed in equal amounts and incubated at room temparture to allow possible assembly of proteins into VLPs. The picture illustrates the non-formation of VLPs.