VIRULENCE CHARACTERIZATION OF RIFT VALLEY FEVER VIRUS STRAINS AND EFFICACY OF GLYCOPROTEIN SUBUNIT VACCINES IN MICE

by

AARON MICHAEL BALOGH

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Dr. Juergen A. Richt

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Abstract

Rift Valley fever virus (RVFV) is a vector-borne zoonotic pathogen endemic to sub-Saharan Africa and the Arabian Peninsula that causes severe disease in ruminants and humans. RVFV is a significant threat to US livestock and public health due to a lack of licensed, efficacious vaccines and its ability to become established in non-endemic areas. Subunit vaccine candidates based on RVFV N- and C-terminal glycoproteins (Gn and Gc) are a viable option for use in ruminants due to their ease of production, safety, and ability to induce immune responses that offer differentiation between infected and vaccinated animals (DIVA). Importantly, subunit Gn+Gc vaccine candidates have demonstrated efficacy in sheep. However, despite the efficacy of a dual glycoprotein vaccine, no studies have directly compared protective efficacies of the individual glycoproteins. Furthermore, although RVFV demonstrates 2.1% maximum pairwise amino acid strain divergence within Gn/Gc ectodomains, it remains unclear how this may affect cross-protective vaccine efficacy. In this study, we used a BALB/c mouse model to determine the median lethal dose (LD_{50}) of 3 wildtype RVFV strains and used this information to standardize challenge doses in subsequent vaccine efficacy studies using baculovirus-expressed Gn/Gc antigens derived from RVFV strain Zagazig Hostpital 1977 (ZH548). Strains Kenya 2006 (Ken06) and Saudi Arabia 2001 (SA01) demonstrated equally high virulence (LD_{50}= 7.9pfu), while recombinant strain South Africa 1951 (rSA51) was less virulent (LD_{50}=150pfu). Following prime-boost vaccination, 100% (10/10) of the Gn+Gc vaccinated mice survived challenge with x1000 LD_{50} Ken06 and SA01, while only 50% (5/10) of Gn+Gc vaccinated mice survived challenge with rSA51. Additionally, 90% (9/10) of Gn-only vaccinated and 40% (4/10) of Gc-only vaccinated mice survived challenge with Ken06. These data suggest that a Gn-only subunit vaccine is an efficacious alternative to dual glycoprotein vaccine candidates and that our ZH548-

derived Gn+Gc vaccine has the potential to cross-protect against divergent RVFV strains. Results from this study can be used to optimize current vaccine formulations and inform future vaccine efficacy and licensure studies in ruminants.

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Dedication

I would like to dedicate this thesis to all the people mentioned above who made this a reality.

Chapter 1 - **Rift Valley Fever Virus Literature Review**

1.1 Introduction

Rift Valley fever virus (RVFV), the causative agent of Rift Valley fever (RVF), is a vector-borne zoonotic virus of the genus *Phlebovirus* in the family *Bunyaviridae* that is endemic to sub-Saharan Africa, Egypt, and countries of the Arabian Peninsula.[1] Since the first confirmed outbreak in 1931 in the Rift Valley of Kenya, RVFV has caused sporadic outbreaks in ruminants and humans, the largest of which occurred in Egypt in 1977-78 in which approximately 200,000 humans developed clinical RVF.[2,3] In ruminants, RVF symptoms range from mild febrile illness to acute hepatitis, encephalitis, and widespread abortions in pregnant sheep and cattle.[1] In humans RVF usually manifests as mild fever but in some cases can progress to hemorrhagic disease, blindness, neurologic disease, and death.[1] RVFV is a significant threat to US livestock and public health due to its potential for introduction into non-endemic areas by malicious or accidental means and is thus considered an overlap select agent by the Centers for Disease Control and Prevention (CDC) and the United States Department of Agriculture (USDA).[4] Despite much effort, there are currently no fully licensed human or veterinary vaccines available outside endemic areas.

The purpose of the studies herein is threefold: 1) to determine the virulence differences between 5 wild-derived and recombinant RVFV strains, 2) to study the interstrain cross-protective efficacy of a recombinant RVFV glycoprotein subunit vaccine, and 3) to consider the relative contribution of RVFV glycoproteins against single strain challenge in mice. The information presented in this introductory chapter consists of a general overview of RVFV with special emphasis on current vaccine constructs, animal models, and interstrain genetic diversity.

1.2 Bunyaviridae Family

Bunyaviridae is a large and diverse family of mainly vector-borne animal viruses consisting of more than 300 species in 5 genera: *Phlebovirus, Hantavirus, Orthobunyavirus, Nairovirus,* and *Tospovirus*. All are characterized as enveloped, tri-segmented, negative or ambisense, single-stranded RNA (ssRNA) viruses with similar genetic content and organization. Many bunyaviruses such as RVFV, Crimean-Congo Hemorrhagic Fever Virus, Hantaviruses, Schmallenberg Virus, and Tomato Spotted Wilt Virus can have significant economic and public health consequences.[5–8]

1.3 RVFV Morphology and Genomic Structure

The RVFV virion is spherical with a T=12 icosahedral symmetry and diameter ranging from 80-120nm (average 100nm).[9] The genome is tripartite consisting of three circular ssRNA segments (Large [L], Medium [M], and Small [S]) encoding 7 proteins with a combined length of ~11.9 kb.[10] The L segment encodes the RNA-dependent RNA polymerase (RdRp).[11] The M segment encodes the non-structural proteins NSm1 (78kDa) and NSm2 (14kDa) and the immunogenic amino and carboxy-terminal surface glycoproteins Gn (G2) and Gc (G1)[12], and the S segment encodes the nonstructural protein NSs and nucleoprotein N.[13]

1.4 RVFV Viral Proteins

1.4.1 Non-structural Protein NSs

NSs is translated from the positive strand of the S-segment vRNA and is considered the primary RVFV virulence factor with a number of defined roles in suppressing host innate immune responses and regulating host transcription.[14–27] NSs primarily functions as a Type I IFN antagonist that complexes to SAP30 and other corepressors and recruits directly the IFN-β

promoter, thereby excluding transcriptional coactivators necessary for IFN-β expression.[18] Additionally, NSs promotes host and viral translation through specific degradation of protein kinase R (PKR) transcription and subsequent inhibition of eukaryotic initiation factor 2 alpha (eIF2α) phosphorylation.[19–21] This is a function that is lacking in other phleboviruses and is thought to contribute to the relatively high pathogenicity of RVFV.[19] Interestingly, although RVFV replicates in the cytoplasm, NSs aggregates form in the nucleus and interact with specific regions of host DNA causing chromosomal segregation defects in infected cells.[24,28,29] These aggregates are unique to RVFV infected cells and are thought to contribute to the fetal malformations seen in infected ruminants.[1] RVFV mutants lacking functional NSs replicate poorly in Type I IFN competent cell lines and have reduced virulence in animal models.[14,30]

1.4.2 M-segment Nonstructural Proteins

RVFV M-segment proteins are encoded from one large open reading frame via differential use of 5 in-frame AUG start codons regulated by a leaky scanning mechanism.[31] The resulting polyproteins are then cleaved by cellular peptidases into individual proteins.[32–35] The translational product from AUG1 is an NSm-Gn-Gc polyprotein that is cleaved in the endoplasmic reticulum resulting in the non-structural NSm-Gn fusion protein known as the 78kDa protein (P78).[10] Although, the exact function of P78 remains unknown it has been shown to be selectively packaged into virus particles derived from mosquito cells but not mammalian cells and is essential for virus dissemination in mosquitoes.[36,37] Translation initiation from AUG2 results in an NSm-Gn-Gc polyprotein in which the structural glycoproteins Gn and Gc are cleaved from NSm resulting in a 14kDa protein that is retained within the cytosolic aspect of the endoplasmic reticulum and eventually trafficked to the outer membrane of the mitochondria where it inhibits virus-induced apoptosis via inhibition of p38 mitogen-activated protein

kinase.[31,38] However, despite this apparent function, NSm is dispensable in mammalian cell culture and does not contribute to virulence in mammals.[38,39]

Previous research suggested that AUG3 does not contribute to M-segment protein expression.[35] However, a recent study indicated the presence of a 13kDa N-terminally truncated NSm protein named NSm' translated from AUG3 and demonstrated that simultaneous deletion of AUG2 and 3, which results in the abrogated expression of NSm and NSm', caused significantly reduced virulence in mice.[37] This is further supported by the fact that the AUG3 protein product can partially compensate for NSm when AUG2 is deleted.[38] AUG4 and 5 contribute modestly to Gn and Gc expression, yet their relative importance to virus replication and virulence has not been determined.[35]

1.4.3 Glycoproteins Gn and Gc

Gn (56kDa) and Gc (65kDa) are surface glycoproteins encoded in the M-segment and are expressed by a leaky scanning mechanism from AUG 2, 4, and 5.[32,35] Gn/Gc are arranged in 5 and 6 subunit heterodimers on the surface of the RVFV virion.[40] Since Gn and Gc are surface exposed, they are the major targets of host immunity, the primary antigen used in vaccine constructs, and the major determinants of host tropism and infectivity. Although limited epitope mapping studies have been conducted, 4 antigenic regions (I–IV) are well conserved across RVFV Gn/Gc except for in some attenuated strains.[41–43]

1.4.4 Ribonucleoproteins Structure and Function

RVFV ribonucleoproteins (RNPs) consist of genomic vRNA, nucleoprotein (N), and the RNA-dependent RNA polymerase (L-protein) and serve as platforms for vRNA replication and transcription.[44] Although the precise structure of the RVFV RNP remains unclear, the mechanism of N and L recruitment to the vRNA has been described.[45,46] N is the most abundant protein in the RVFV virus particle and is a major immunogen in natural infection and target for

differentiation between infected and vaccinated animal (DIVA) compatible vaccines.[47–49] N forms hexameric ring-like structures that closely associate with vRNA and play key roles in RNA replication, transcription, virus assembly, and protection from innate immune responses.[44,50] The L-protein is a large multi-domain polymerase responsible for replication and transcription of vRNA.[11,51,52] Although functional motifs (motifs 1-3) have been described for RVFV L that resemble other segmented and non-segmented viruses limited functional studies have been performed.[11] Further structural analysis of RVFV RNPs and its components will be beneficial for the development of potential antiviral drugs.

1.5 Replication Cycle

Although the complete RVFV replication cycle has not been fully elucidated, reverse genetics, non-spreading virus particles, and minigenome systems have helped define critical processes of attachment, RNA synthesis and packaging.[53,54]

1.5.1 Virus Attachment and entry

RVFV attachment and entry in most cells is mediated by heparan sulfate, but attachment and entry into dermal dendritic cells at the site of initial infection (i.e. skin) is mediated by dendritic cell-specific intercellular adhesion molecule 3- grabbing nonintegrin (DC-SIGN).[55,56] After attachment, entry occurs via a dynamin-dependent caveola-mediated mechanism followed by membrane fusion by a type II pH dependent mechanism mediated by a Gc conformational change and uncoating in the late endosome.[31,57,58]

1.5.2 Genome Replication and Transcription

Once the virion is uncoated in the cytosol, each segment is immediately transcribed into mRNA or replicated into complimentary RNA (cRNA) as an exact genomic copy by the L-protein using a 5’ nucleoside triphosphate. Since the S segment is ambisense, the NSs cRNA is contained in the virion and can be expressed early, which makes evolutionary sense because NSs

is involved in suppressing innate immunity.[10] Like all other segmented negative strand RNA viruses, bunyaviruses employ a "cap-snatching" mechanism of transcription initiation to acquire 5' caps from host mRNA via the endonuclease activity of the L-protein.[59–61] However, unlike Influenza virus, which cap-snatches and replicates in the nucleus, bunyaviruses cap-snatch and replicate in the cytoplasm.[62] Although the RVFV L-protein is responsible for both replication and transcription, the sequence motifs involved in each process are not defined.[11]

1.5.3 Virus Assembly and Release

Once translated and cleaved from other M segment proteins, Gn and Gc localize to the Golgi apparatus independently of other viral proteins via a localization signal present on Gn.[63,64] Other core virion proteins are recruited to the Golgi by unknown signals and mechanisms.[10] Virions formed within the Golgi are then transported in vacuoles to the cell surface where they fuse with the cell membrane and exit the cell.[10]

1.6 RVFV Ecology and Vectors

RVFV outbreaks are sporadic yet predictively follow periods of El Niño and heavy rainfall, which induces hatching of infected mosquitos from flooded soils.[65–67] Although many RVFV vector species have been documented in experimental and natural settings, the vectors most closely associated with RVFV maintenance and transmission are *Aedes* and *Culex* species mosquitos. RVFV mosquito vectors are divided into two groups depending on their role in the transmission cycle. "Reservoir/maintenance" vectors (primarily *Aedes spp.*) are associated with transiently flooded areas while "epidemic/amplifying vectors" (primarily *Culex spp*) vectors are associated with permanent water bodies.[68,69] Upon hatching, infected RVFV mosquitos feed on nearby wild or domestic ruminants. Subsequent transmission occurs when naïve mosquitos feed upon viremic hosts and transmit the virus to naïve hosts. Importantly, the presence of competent

vectors in Europe and the United States demonstrates the potential for global spread and establishment of RVFV.[70,71]

Although RVFV is characterized by sporadic outbreaks in domestic livestock and humans, seroprevalence and virus isolation studies in Kenyan mammals and mosquitos indicate that RVFV circulates in a cryptic enzootic cycle during interepidemic periods.[72–77] Thus, enhanced surveillance programs may be implemented to more accurately predict RVFV outbreaks.

1.7 Genetic Diversity of RVFV

The genetic diversity of RVFV is low, with Bayesian estimates of maximum inter-strain pairwise divergence of 5% and 2% at the nucleic and amino acid levels, respectively.[41] This is likely because the time to most recent common ancestor (TMRCA) for RVFV is recent, with an estimated time of 120-130 years.[41,78] Another explanation for this low divergence is the so-called "double-filter" hypothesis, which suggests that arbovirus genomes are subject to selective pressures in both the mammalian and insect host, thus leading to tighter genomic constraint.[41,79] This hypothesis has been supported for RVFV *in vitro* in which the genomic stability of *NSs* was dependent upon alternative passage between insect and mammalian cells.[80] However, genetic diversity in RVFV is lower compared to other arthropod-borne bunyaviruses such as Crimean-Congo Hemorrhagic Fever (CCHF), which lends support to the recent TMRCA hypothesis.[78] Despite this low diversity, phylogenetic patterns can be inferred as RVFV strains generally cluster based on geographic location.[78,41]

Interestingly, surface exposed epitopes of Gn/Gc show low nucleic acid sequence diversity (maximum 5-6%) despite being targets of neutralizing antibodies.[41] In contrast, the intracellular and untranslated regions of Gn/Gc show up to 15% nucleic acid sequence

divergence.[41] Sequence diversity for both the S and L segment are also low, with most sequence differences located in intergenic regions.[41]

RVFV genomic reassortment has been experimentally demonstrated in mammalian cell culture and live mosquitoes and phylogenetic analysis suggests it occurs in nature.[81,82,78,83] Reassortment events raise safety concerns when using live-attenuated vaccines and may play a role in RVFV evolution.[78]

Contrary to the low genetic diversity of RVFV, interstrain variation in virulence among wild-derived and recombinant strains is high.[41,43] One study determined the LD_{50} values (plaque forming units [pfu]) of 21 RVFV strains in outbred ICR and inbred C57BL/6 mice varied from <1 to $1.3x10^6$ pfu.[43] A similar study conducted in adult inbred Wistar-Furth (WF) rats also indicated interstrain variation in RVFV virulence.[84,85] Interestingly, RVFV isolates in Lineage A (Egypt 1977-1979) were more pathogenic in WF rats than representative strains from sub-Saharan lineages[85,86]. This, coupled with the fact that the Egypt 1977 outbreak was the largest RVFV outbreak in history indicates that geographic spread of RVFV to non-endemic areas may increase the virulence of the resulting outbreak strains. Additionally, specific loci have been found, which support these virulence differences.[41]

1.8 Disease in Animals and Humans

Early experiments to determine RVFV host range showed that non-ruminants such as birds, horses, rabbits, and pigs were resistant to RVFV but that mice, rats, and hamsters were highly susceptible.[87] Since then, mice and rats have been the main platform to conduct RVFV pathogenicity and vaccine efficacy studies, while RVF in livestock has mostly been characterized anecdotally during outbreaks with some laboratory-based experimental studies. Limited studies have been conducted in non-human primate models.

1.8.1 Mice

Mice are the most widely used animal model to study RVFV pathogenesis and vaccines because they are highly susceptible, cost-effective, and mirror disease seen in ruminants and humans.[1] Mice show severe clinical signs by 2-3dpi characterized by ruffled fur, hunched posture, and lethargy and usually succumb to fulminant hepatitis in 2-5 days post infection (p.i.).[88] However, depending on inoculation route, some survive this stage and succumb to encephalitis on day 8-14dpi.[88,89] Clinical disease onset is correlated with viral titers in the serum liver, and spleen and a significant decrease in blood platelets indicative of hemorrhagic disease.[88,89] Despite decreases in blood platelets however, mice do not exhibit extreme hemorrhagic manifestations.[1] Furthermore, mice infected with wild-type RVFV show temporal increases in pro-inflammatory cytokines that is thought to contribute to disease severity.[89] In addition to viral determinants of disease, host factors are thought to play a role in influencing disease severity, especially those genes involved in regulating the activation of the Type I IFN pathway.[90,91]

1.8.2 Rats

Unlike mice, rats (*Rattus norvegicus*) show varied RVFV susceptibility similar to that of humans and ruminants making them more appropriate animal models.[84] Symptoms in susceptible strains such as Wistar-Furth are characterized by fatal hepatic disease and death as early as two days, while the more resistant Lewis strain has a significantly higher survival rate but can be more susceptible to encephalitis at later time points.[92] However, like mice, rats do not develop hemorrhagic manifestations in response to RVFV infection.[1]

1.8.3 Ruminants

Ruminants are the most applicable model for studying RVFV pathogenesis and vaccines as they are the natural host of RVFV. However, lack of large animal biocontainment facilities

can limits their usefulness. Despite this, RVFV infection in sheep has been studied in both laboratory and natural settings. Neonatal and newborn lambs are most susceptible to RVFV infection as demonstrated by so-called "abortion storms", in which rapid virus transmission induces widespread abortions in herds of pregnant sheep causing up to 100% neonatal mortality.[83,93] Although RVF in neonatal and young lambs is nearly uniformly fatal, adult sheep are differentially susceptible depending on RVFV strain and sheep breed.[3,94–97]

Other ruminants such as goats and camels are less susceptible to RVFV, though experimental and observational data show cattle can develop severe clinical disease characterized by febrile illness and high fetal mortality.[87,98,99] Interestingly, the gradual replacement of native more resistant livestock (such as Zebu cattle) with more susceptible European breeds is thought to contribute to the increase of RVFV outbreaks throughout Africa.[41,93] Although some studies have been performed in cattle, more work is needed to better characterize the pathogenesis and efficacy of candidate vaccines in this model.[100,101]

1.8.4 Non-human Primates

Although most non-human primates show mild clinical RVF, rhesus macaques best demonstrate the range of symptoms seen in humans.[102] In macaques infected intravascularly, symptoms range from mild febrile illness to severe disease characterized by anorexia, lassitude, vomiting, and hemorrhagic manifestations.[102] However, newer models need to be developed that mimic the natural extravascular inoculation route of RVFV by mosquitoes.[103]

1.8.5 Humans

RVF in humans has been studied during large outbreaks such as the Saudi Arabian outbreak of 2000-01 and through cases of laboratory acquired infections in the 1930s-40s.[104–106] Symptoms in humans are usually characterized by self-limiting febrile illness but can progress to neurological complications, blindness, thrombosis, and hemorrhagic fever. Death normally

occurs in 1-2% of patients but varies widely by outbreak.[1] Although increased abortion rates are not seen during RVFV outbreaks, vertical transmission has been documented in one case leading to the death of a newborn.[106]

1.9 RVFV Vaccines

Many RVFV vaccines such as formalin-inactivated, live-attenuated, virus-vectored, subunit, and DNA constructs have been developed and tested, to date yet none are approved for use in humans or animals outside endemic areas.[49] Although cell-mediated immunity has been measured for only a few RVFV vaccines, some studies suggest that humoral immune responses are sufficient for protection against RVFV.[49,107,108]

Early RVFV vaccines were made from inactivated material such as the mosquito-isolated Entebbe strain from Uganda.[109] Although this vaccine is efficacious in humans, the multiple dose requirement limits its applicability in resource-limited agricultural settings.[110,111]

Live-attenuated vaccines provide a cost-effective alternative to inactivated vaccines and some constructs have conditional or full licensure in the United States and African countries.[49] Clone 13 is a naturally attenuated RVFV strain isolated in Central Africa from an infected patient that has a 70% deletion in the NSs gene.[112] Importantly, unlike other live-attenuated RVFV vaccines such as the Smithburn strain, Clone 13 induces protective immunity and does not cause abortions or fetal malformations in sheep.[113] Although Clone 13 is attenuated, other NSs-deficient strains can cause severe neurological disease in mice after intranasal inoculation suggesting that some virulence is still retained in strains lacking NSs.[114] Importantly, although Clone 13 is attenuated in cell culture and animal models, it is not exempt from the federal select agent list restricting its use in research to biocontainment laboratories.

Mutagenized passage 12 (MP-12) is a live attenuated vaccine strain derived from virulent strain Zagazig Hostpital 548 (ZH548) after 12 successive cell culture passages in the presence of

chemical mutagen 5-fluorouracil.[115] MP-12 is efficacious as a vaccine in both humans and livestock, causes minimal teratogenicity in livestock, and is attenuated in all 3 strains limiting the chances of wild-type reversion.[99,116,117] MP-12 is advantageous for research because reverse genetics methods have been established and it is exempt from the federal select agent list making it conducive to mutation experiments outside biocontainment laboratories.[30] In general, live-attenuated vaccines are efficacious, but issues with potential reversion to virulence and recombination with wildtype strains limits their usefulness mostly to outbreak settings in endemic areas.

A number of recombinant vaccines have been developed for RVFV including subunit and virus-vectored.[49] Recombinant vaccines are advantageous due to their ability to induce targeted immunity to specific proteins and epitopes, inability to revert to wild-type, and differentiation between immunized and vaccinated animals (DIVA) compatibility. Thus, these vaccines may be more useful in non-endemic areas containing serologically naïve host species. Recently, a highly immunogenic baculovirus-expressed Gn ectodomain/Gc (Gne/Gc) subunit vaccine was developed that induced protective immunity against lethal RVFV challenge in sheep.[118,119,120] Unlike other subunit vaccine constructs tested, this vaccine induced potentially protective immunity after a single dose.[121–123] Ideal RVFV vaccines should elicit protective immunity after one dose, be DIVA compatible, and cheap to produce.

1.10 Purpose of Research

RVFV is a major threat to US agriculture and public health and currently no licensed vaccines are available for human or animal use outside of endemic areas. Based on low genetic diversity between RVFV strains, it is assumed that current vaccine candidates will cross-protect against all RVFV strains. However, with the emergence of new outbreak strains and continued divergence of the RVFV genome, vaccine constructs based on old RVFV genotypes may not be

effective in the future. Using a BALB/c mouse model the purpose of this study was 2-fold, (1) to evaluate the cross-protective efficacy of a recombinant Gn+Gc subunit vaccine candidate, and (2) to evaluate the individual protective efficacies of Gn and Gc. Results from this study can be used to inform the development of RVFV vaccines and livestock vaccine-challenge models.

Chapter 2 - **Materials and Methods**

2.1 Cells

VeroE6 cells were cultured in 1x Dulbecco's Modified Eagle's Medium (DMEM) or 1x MEM with 10% fetal bovine serum (FBS). Baby Hamster Kidney T7/9 (BHKT7/9) cells were cultured in MEM alpha with 10% FBS with or without hygromycin B. Medical Research Council 5 (MRC-5) cells were cultured in 1x MEM with 10% FBS. All cells were incubated at 37^{o}C with 5% CO_2.

2.2 Viruses

Wild derived RVFV strains 128B-15 (Ken06) and Saudi Arabia 2001 (SA01) were acquired from Dr. Barry Miller (Centers for Disease Control and Prevention) via Dr. Richard Bowen (Colorado State University) and recombinant RVFV strains South Africa 1951 (rSA51) was rescued using the reverse genetics protocol described below. All viruses were propagated in VeroE6 cells in the culture conditions described above.

2.3 Plaque Assay

RVFV stocks and tissue-derived virus were titrated via plaque assay. Briefly, virus was diluted 10-fold in MEM containing 10% FBS. 250ul of each dilution was plated onto confluent VeroE6 cells in a 12-well plate. After one hour of incubation, the supernatants were removed and monolayers were covered with ~1mL of semi-solid overlay (1:1 ratio 2xMEM w/10% FBS, 1% antibiotics and methyl cellulose). After 4-5 days incubation, cells were stained with ~500uL of 1% crystal violet fixative and incubated at room temperature for 1hr. The plates were then washed with a stream of cool water and allowed to air-dry before plaque enumeration.

2.4 Reverse Genetics

rSA51 was rescued using methods previously described.[124] Briefly, 3 plasmids encoding full-length L, M, and S genomic segments and 3 protein expression plasmids encoding Gn/Gc glycoproteins, nucleoprotein N, and L polymerase were transfected onto BHKT7/9 cells cultured in MEM alpha containing hygromycin. After 24 hours, the media was removed and replaced with fresh MEM alpha without hygromycin. After 5 additional days, supernatants were transferred onto confluent VeroE6 cells and monitored daily for cypopathic effect (CPE). Supernatants from CPE positive cells were labelled as passage 0 (P0) and stored at -80^{o}C.

2.5 RNA Extraction and Sequencing

RNA from all virus strains were inactivated, extracted, and sequenced as previously described.[125] Briefly, virus supernatants were mixed 1:4 in TRIzol-LS reagent (Life Technologies, MD) to ensure proper inactivation before removal from containment. RNA was then extracted using the RNeasey minikit (Qiagen, CA) via the manufacturer's instructions. Each genomic segment (L, M, and S) was amplified in 2 segments using a T7 ligase-based system and subsequently reverse transcribed using SuperScript III first-strand synthesis SuperMix (Invitrogen, CA). After preparation of the sequencing library using the Nextera XT-DNA kit (Illumina, CA), sequencing was performed using 150 base pair paired-end reads. Resulting consensus sequences were compared to reference sequences in GenBank (Table 2-1).

Table 2-1 GenBank accession numbers for RVFV strains Ken06, SA01, and SA51

Strain	L-segment	M-segment	S-segment

	Accession #	Accession #	Accession #
Ken06 (128B-15)	KX096938.1	KX096939.1	KX096940.1
SA01 (SA01-1322)	KX096941.1	KX096942.1	KX096943.1
SA51	DQ375433.1	DQ380195	DQ380158.1

Table 2-1. GenBank accession numbers for the L-, M-, and S-segments of RVFV strains Ken06, SA01, and SA51

2.6 Analysis of Viral Growth

To determine the growth kinetics of wildtype RVFV strains in cell culture, 0.01 MOI of RVFV strains were diluted in 1xMEM containing 10% FBS and inoculated onto triplicate wells of confluent MRC5 cells in a 24-well plate. After attachment for 1h cells were washed once with sterile virus diluent before 500µl media was added. Supernatants were collected at 0, 24, 48, 72, 96 hours post infection (hpi) and titrated via plaque assay.

2.7 LD_{50} Determination

To quantify the virulence of all challenge strains, groups of 5 6-8 week old female BALB/c mice were (Charles River Laboratories, Wilmington, MA, USA) challenged subcutaneously with 10-fold serial dilutions of RVFV ranging from 1000pfu to 0.1pfu diluted in 200ul of serum-free MEM (Table 2-2). Negative control mice were mock challenged with 200ul of serum-free MEM without virus. Mice were monitored 3x daily for clinical signs for a maximum of 12 days and euthanized when moribund according to pre-defined clinical criteria. Culled and found-dead mice were necropsied and tissues collected for downstream analysis. During the challenge period, all mice were housed in biosafety level 3(BSL-3) containment at the Biosecurity Research Institute (BRI, Kansas State University, Manhattan, KS). All protocols were approved by the Institutional Animal Care and Use Committee (IACUC).

LD_{50} values were calculated using the Spearman-Karber method (Microsoft Excel) and directly compared using Log-Rank test in Graphpad Prism program (GraphPad Software Inc., La Jolla, CA).[126] Statistical significance was defined as $P<0.05$.

Table 2-2 LD_{50} Experimental Design

	Dose (pfu)					
	0.1	1	10	100	1000	
RVFV Strain	**Number of Mice**					**Total**
Ken06	5	5	5	5	5	25
SA01	5	5	5	5	5	25
rSA51	5	5	5	5	5	25
rZinga	5	5	5	5	5	25
vKen06	5	5	5	5	5	25
MP-12	5	5	5	5	5	25
Mock	-	-	-	-	-	20

Table 3-1 Description of the LD_{50} experimental study design. Five mice for each of the 5 dilution groups were used.

2.8 Tissue Homogenization

Portions of tissues were added to sterile 1.5mL microcentrifuge tubes with sterile 1x MEM and 1-2 steel beads. Following homogenization in a TissueLyser (Qiagen), homogenates were centrifuged at 8000xg for 5 min. Then, supernatants were harvested and stored at -80^{o}C until titrated.

2.9 Vaccination and Challenge

Baculovirus expressed, affinity purified recombinant Gn and/or Gc antigen derived from RVFV strain ZH548 was emulsified in a 3:1 ratio with Montanide ISA25 VG oil-in-water adjuvant (Seppic, France) via 4x passage through a 3/8" 26g needle as described previously[119]. Then, 6-8 week old female BALB/c mice (Charles River Laboratories, Wilmington, MA, USA) were vaccinated subcutaneously with either 5ug each Gn and Gc or 10ug Gn or Gc only in a total volume of 200ul. On day 21 post first vaccination (dpfv) mice were boosted with the same

antigen(s) as per prime vaccination. Five days before challenge, mice were moved to a biosafety level 3 vivarium at the BRI to allow for adequate acclimation. Blood was collected via the lateral saphenous vein using a 26g needle and capillary tube on 0, 21, and 35dpfv. On day 35dpfv, mice were challenged subcutaneously with x1000 LD_{50} of RVFV. Animals were monitored 3x daily until 12 days post challenge (dpc) as above. On 3dpc a subset of mice were culled and necropsied and whole liver, spleen, and brain were collected for downstream analysis. All protocols were approved by the KSU IACUC committee as per above.

Table 2-3 Vaccine-Challenge Study Design

Challenge Strain	Vaccine	Vaccine Dose	Challenge Dose (pfu)	Number of Mice
Ken06	Gn+Gc	5ug each	8000	10
	Gn	10ug	8000	10
	Gc	10ug	8000	10
	Mock	None	8000	10
SA01	Gn+Gc	5ug each	8000	10
	Mock	10ug	8000	10
rSA51	Gn+Gc	10ug	150000	10
	Mock	None	150000	10

Table 2-3. N=10 mice per group were vaccinated with 5ug each Gn+Gc or 10ug total Gn- or Gc-only and boosted with the same antigen and dose on 28dpfv. Mice were then challenged on 35dpfv with x1000 LD_{50} of RVFV.

2.10 Enzyme Linked Immunosorbent Assay (ELISA)

A previously described ELISA assay was used to measure antibody titers in mice serum on 0, 21, and 28dpfv.[118] Briefly, 96-well polystyrene (Nunc, Maxisorp) plates were coated overnight at 4c with 200ng recombinant Gn or Gc in 100ul Dulbecco's coating buffer (pH 7.3). Plates were then blocked with PBS (pH 7.3) with 1% skim milk and 0.1% Tween-20 for 15min at 37C. Plates were then washed 3x with PBS with 0.1% Tween-20. Then, 100ul of test serum diluted 1:50 in blocking buffer was added in duplicate to the plate and incubated at 37C for 1h. After washing 3x, 100ul Protein-G-HRP (Abcam, Cambridge, MA) diluted 1:50000 in blocking

buffer was added to each well and incubated for 1h at 37C. After washing 3x, 100ul of substrate containing 1:1 mixture of 0.1mg/mL 3,3'5,5'-tetra-methylbenzidine (TMB) (Thermo Scientific, Rockford, IL) and H_2O_2 was added to each well and allowed to incubate at room temperature in the dark for 25min. 100ul of H_2SO_4 was added to the wells to stop the reaction. Optical densities (OD) were read at 450nm on a microplate reader (Fluostar Omega, BMG Labtech, Cary, NC).

2.11 Plaque Reduction Neutralization Test 80% ($PRNT_{80}$)

$PRNT_{80}$ assays were performed as described previously.[119,120] Briefly, mouse serum was diluted two-fold from 1:20 to 1:1280 in 1xMEM with 2% bovine serum albumin (BSA) and 1% antibiotics. Diluted serum was then combined 1:1 with 50pfu RVFV (MP-12) in 4% BSA in a total volume of 500ul and incubated for 1h at 37C. The virus-serum mixture was then plaque assay titrated as per above.

2.12 Statistical Analysis

Analyses for significant differences between survival curves were performed via Log Rank test (Mantel-Cox). One-tailed T-tests were used to compare tissue titers. Differences in virus replication in the one-step growth curves were analyzed by one-way ANOVA in GraphPrad Prism.

Chapter 3 - **Results**

3.1 Sequence Analysis of Wildtype RVFV Strains

To determine the sequence similarity between the 3 wildtype RVFV strains used in this study, we aligned the deduced amino acid (AA) sequences of each strain and calculated the pairwise percent identity and total number of AA point mutations between the L, M, and S segments of each strain (Tables 3-1, 2, 3). Overall, high conservation was seen between the three strains for each segment with pairwise strain similarity ranging from 98.63% (S-segment SA01 vs SA51) - 99.48% (L-segment Ken06 vs SA01). The greatest total number of substitutions within a gene segment was seen in the L-segment between SA01 and SA51 (13 substitutions) and the lowest was seen in the S-segment between Ken06 and SA51 (4 substitutions). Within the M-segment, multiple substitutions were seen within the NSm, Gn, and Gc amino acid sequences among all strains compared (Table 3-2). Within the S-segment, all substitutions were seen within the NSs amino acid sequence. The nucleoprotein (N) amino acid was completely conserved (Table 3-3).

Table 3-1 L-segment amino acid sequence comparison between RVFV strains Ken06, SA01, and SA51

L segment AA Comparisons

Number	Ken06 vs. SA01	Ken06 vs. rSA51	SA01 vs. SA51
	Similarity (%)		
	99.48	**99.39**	**99.3**
1	V164I	R244K	I164V
2	F291L	K249R	R244K
3	S411G	I349V	K249R
4	N435D	G407D	L291F
5	L440P	A426G	I349V
6	K493R	N435D	G407D
7	T577A	L440P	G411S
8	K947R	N446D	A426G
9	S1656N	K493R	N446D
10	S1670P	K947R	A577T
11	A2001T	N1724S	N1656S
12		A2001T	N1670S
13			N1724S

Table 3-2 M-segment amino acid sequence comparison between RVFV strains Ken06, SA01, and rSA51

M segment AA Comparisons

Number	Ken06 vs. SA01	Ken06 vs. rSA51	SA01 vs. SA51
	Similarity (%)		
	99.08	**99.25**	**98.9**
1	E118G (NSm)	V60I (NSm)	V60I (NSm)
2	A123T (NSm)	N95D (NSm)	N95D (NSm)
3	Y433F (Gn)	H408R (Gn)	G118E (Nsm)
4	I589V (Gn)	I589V (Gn)	T123A (NSm)
5	T595I (Gn)	T595V (Gn)	H408R (Gn)
6	V602I (Gn)	V602I (Gn)	F433Y (Gn)
7	A659V (Gn)	R605K (Gn)	I595V (Gn)
8	N662D (Gn)	I863V (Gc)	R605K (Gn)
9	V685I (Gn)	V954I (Gc)	V659A (Gn)
10	V954I (Gc)		D662N (Gn)
11	D987E (Gc)		I685V (Gn)
12			I863V (Gc)
13			E987D (Gc)

Table 3-3 S-segment amino acid sequence comparison between RVFV strains Ken06, SA01, and rSA51

S segment AA Comparisons

Number	Ken06 vs. SA01	Ken06 vs. rSA51	SA01 vs. SA51
	Similarity (%)		
	99.02	**99.22**	**98.63**
1	V62A	Y3F	Y3F
2	V189I	K24R	K24R
3	R202K	R202K	A62V
4	I239V	G253E	I189V
5	M250I		V239I
6			I250M
7			G253E

Tables 3-1, 2, and 3. Amino acid sequence comparison of strains Ken06, SA01, and SA51. Percent similarity refers to the percent amino acid substitution rate for entire gene segment. (3-1) Comparison of L-segment amino acid sequence. Numbers within each amino acid substitution represent the amino acid position starting from the beginning of the L polymerase coding region. (3-2) Comparison of M-segment amino acid sequence. Numbers within each amino acid substitution represent the amino acid position starting from the beginning of the NSm coding region. Designations in parentheses represent corresponding protein in which the mutation is located. (3-3) Comparison of S-segment amino acid sequence. Numbers within each amino acid substitution represent the amino acid position starting from the beginning of the NSs coding region. No amino acid substitutions were found in the nucleoprotein (N) coding region.

3.2 One-step Growth Curve Analysis of Wildtype RVFV Strains

Because Ken06, SA01, and rSA51 are genetically distinct strains of RVFV, we analyzed virus growth kinetics in type I interferon (IFN) competent MRC5 cells using starting inoculum of 0.01 MOI (Figure 3-1). Replication between strains Ken06 and rSA51 were similar at all time points. However, titers of SA01 were significantly less than strains Ken06 and rSA51 at 24 ($P<0.0001$), 48 ($P=0.0017$), 72 ($P=0.0255$), and 96 ($P=0.0052$) hours post infection (hpi). This suggests that SA01 is less capable of replication in MRC5 cells at MOI 0.01 compared with Ken06 and rSA51.

Figure 3-1 Growth Curve of Wildtype RVFV Strains in MRC5 Cells

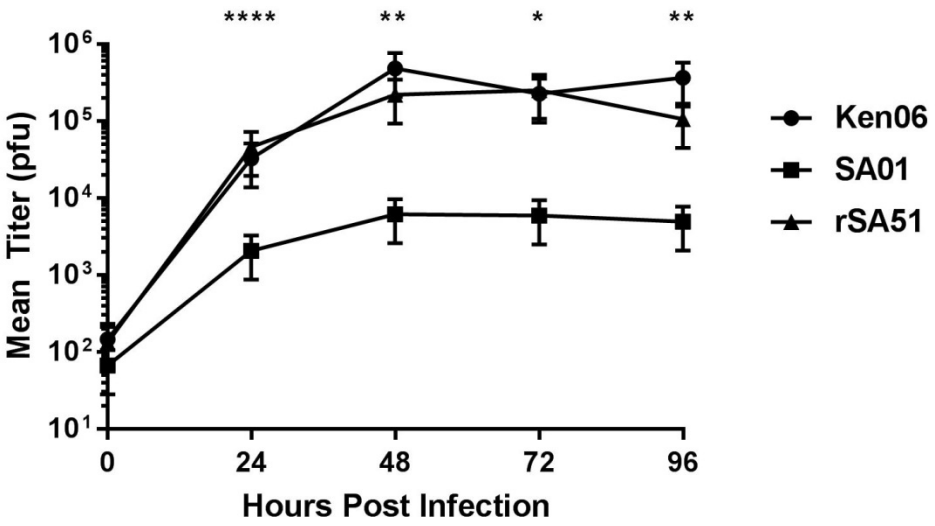

Fig 3-1. MRC5 cells were infected with 0.01 MOI of RVFV in triplicate wells. Supernatants were collectedat 0, 24, 48, 72, and 96hpi and subsequently titrated via plaque assay. Each data point represents the mean of 3 independent experiments, and error bars represent standard error of the mean (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001).

3.3 LD_{50} Determination of Virulent WT RVFV Strains

In order to standardize challenge doses for downstream vaccine-challenge experiments, we determined the LD_{50} of Ken06, SA01, and rSA51 using a BALB/c mouse model. Five mice per dilution group were challenged subcutaneously with 10-fold serial dilutions of RVFV according to Table 2-1 and monitored for survival for 10 days. All challenge groups demonstrated dose-dependent survival excluding the attenuated vaccine strain MP-12, which was used as a negative control in this study (Figure 3-2). Strain-to-strain variation in virulence was apparent with calculated LD_{50} values calculated as 7.9pfu for Ken06 and SA01 and 149.8pfu rSA51 (Table 3-4). Additionally, mean time to death averaged across all doses per strain was associated with increasing LD_{50} values. Thus, our data suggests that RVFV strains Ken06 and SA01 are more virulent than rSA51.

Table 3-4 LD_{50} values and mean time to death

RVFV Strain	LD_{50} (PFU)	Mean Time to Death (days)
Ken06	7.9	3.8
SA01	7.9	2.75
rSA51	149.8	4.33
MP-12	>1000	N/A

Table 3-4. Summary of LD_{50} and mean time to death of various RVFV strains.

Figure 3-2 LD_{50} Survival Curves

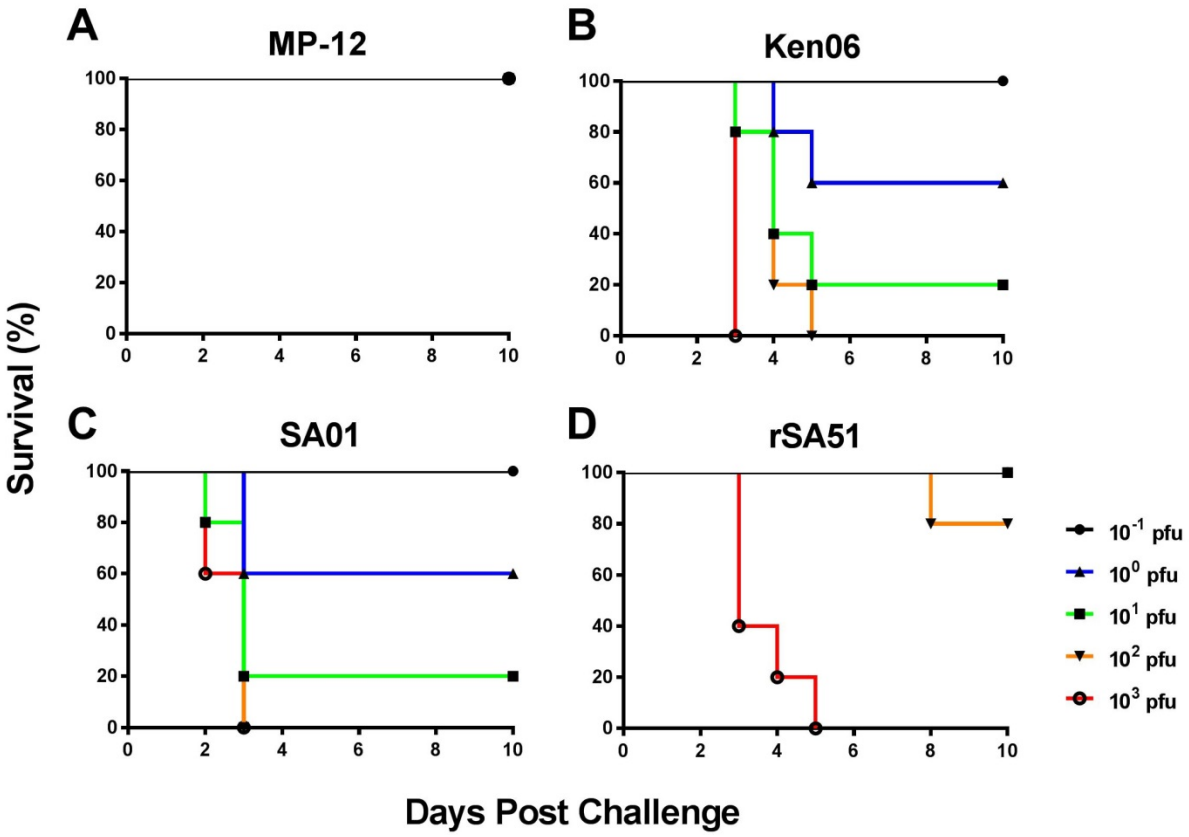

Fig 3-2. Dose dependent survival of mice infected with RVFV strains. Groups of 5 female BALB/c mice were challenged subcutaneously with increasing doses of 10^{-1}, 10^{0}, 10^{1}, 10^{2}, or 10^{3} pfu of RVFV strain MP-12, Ken06, SA01, or rSA51and monitored for survival 3x daily for 10 days.

3.4 Liver titers of mice infected with RVFV strains

To confirm and compare virus replication of RVFV strains in a key target organ, we titrated liver homogenates of all mice within the LD_{50} study challenged with 1000pfu RVFV. Virus replicated efficiently in the liver of all mice tested however replication levels differed significantly between strains (Figure 3-3). Mean liver virus titers of Ken06-infected mice ($4.9x10^5$ PFU/mL) were significantly lower than mean liver virus titers of both rSA51-infected ($9.0x10^6$ PFU/mL, P=0.007) and SA01-infected mice ($4.6x10^6$, P =0.0497). However, no significant difference in liver titers were seen between SA01 and rSA51 infected mice (P=0.12). These data suggest that there are strain-to-strain differences in RVFV replication kinetics in mice.

Figure 3-3 Virus liver titers of mice challenged with 1000 PFU RVFV strains

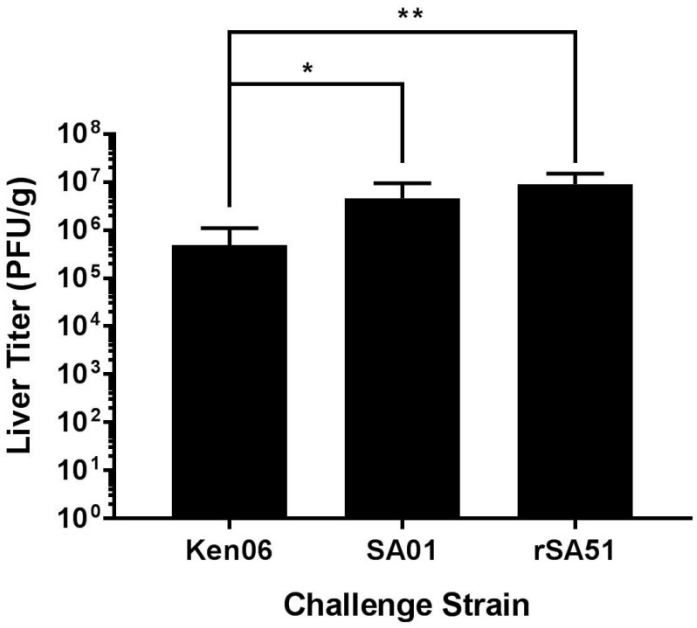

Fig 3-3. Liver homogenates from 1000 PFU virus challenge groups for strains Ken06, SA01, and rSA51 were titrated via plaque assay. Each bar represents the mean virus titer of 5 independent liver homogenates, and error bars represent standard error of the mean (*, P<0.05; **, P<0.01).

3.5 Comparison of Gn and Gc AA sequences between vaccine and challenge strains

The subunit vaccine antigens used in this study were derived from RVFV strain ZH548. To infer the level of potential cross-protection between vaccine and challenge strain antigens, we determined the amino acid sequence similarity of Gn and Gc between ZH548 and challenge strains SA01, Ken06, and SA51. In total compared to ZH548, 8 substitutions were found within the Gn and Gc amino acid sequences of SA01, and 9 each were found for Ken06 and SA51. To determine the relevance of these mutations in the context of vaccine cross-protection, we quantified substitutions within the Gn and Gc ectodomains – the domains demonstrated to elicit neutralizing antibodies. Within the ectodomains SA01 demonstrated 3 substitutions in Gn (L232Q, Y433F, D566G) and 2 in Gc (D987E, S1059T); Ken06 demonstrated 2 substitutions in Gn (L232Q, D566G) and 2 in Gc (I954V, S1059T); and SA51 demonstrated 3 substitutions Gn (L232Q, H408R, D566G) and 2 in Gc (I863V, S1059T). Within Gn, Y433F of SA01 and H408R of SA51 were unique to those strains. In contrast, L232Q and D566G were shared among all 3 challenge strains. Within the Gc ectodomain, D987E was unique to SA01, I954V was unique to Ken06, and I863V was unique to SA51. In contrast, S1059T was common to all strains.

To put these data into a broader context, we compared the Gn and Gc amino acid sequence of ZH548 to all known RVFV strains currently available on GenBank in an effort to determine which strain is most divergent from ZH548 within Gn and Gc. Based on our alignment, strain Ken/Gar-004/06 (M-segment accession #AEB20462., ref. 41) demonstrated the greatest Gn and Gc amino acid sequence divergence from strain ZH548. Compared to ZH548, Ken/Gar-004/06 had 18 total mutations, 15 of which were located within the Gn and Gc ectodomains.

Additionally, we sought to determine the sequence divergence within 3 known virus neutralizing epitopes within Gn.[127] Amino acid sequence within these regions were extremely

conserved across strains with 100% conservation seen between ZH548 and Ken06, SA01, and rSA51. Compared to ZH-548Six strains had single amino acid substitutions in epitope I, 6 strains had 1-2 amino acid substitutions in epitope II, and 1 strain had an amino acid substitution in epitope III (Table 3-6). No strain had amino acid substitutions in all 3 epitopes.

Table 3-5 Gn and Gc amino acid sequence comparison of vaccine antigens to challenge strain antigens

Number	SA01	Ken06	SA51	Ken/Gar-004/06
1	L232Q	L232Q	L232Q	L232Q
2	Y433F	D566G	H408R	K352Q
3	D566G	V589I	D566G	S410R
4	I631V	I595T	I595V	C413R
5	N662D	I602V	R605K	H436R
6	V685I	I631V	I631V	G449R
7	D987E	V659A	V659A	D566G
8	S1059T	I954V	I863V	Y577N
9		S1059T	S1059T	C579S
10				T581S
11				V589I
12				I595T
13				I602V
14				I631V
15				V659A
16				I954V
17				M1014R
18				S1059T

Fig 3-3. Comparison of Gn and Gc amino acid sequences between challenge strains and vaccine strain. Highlighting colors correspond to specific regions within the Gn and Gc protein: Yellow = Gn ectodomain; Green = Gn intermembrane domain; Teal = Gn cytosolic domain; Purple = Gc ectodomain. Text color indicates whether mutation is unique to one of the four strains analyzed: Black = not unique to strain; red = unique to strain.

Table 3-6 RVFV strains with mutations within known neutralizing epitopes in Gn

Strain	Position	Epitope	M segment Accession number
2007004194	N261S	I	EU574031.1
HV-B375	Y275H	I	DQ380218

HB1752	Y275H	I	KJ782453.1
763/70	E276G	I	DQ380188
ArB1986	Q286L	I	KJ782456.1
Zinga	Q286L	I, III	DQ380217
ANK-3837	T384K	II	DQ380215
ANK-3837	M385L	II	DQ380215
ANK-6087	T384K	II	DQ380216
ANK-6087	M383L	II	DQ380216
Lunyo	D386V	II	KU167026.1
ZH-501-777	S387P	II	DQ380202.1
Entebbe	R391I	II	DQ380191
Kenya 57 (Rintoul)	R391I	II	DQ380192.1

Table 3-6. Summary of known mutations within 3 known Gn-specific neutralizing epitopes.

3.6 Recombinant Gn+Gc vaccination induces a detectable immune response and differentially protects mice against challenge with Ken06, SA01, and rSA51

Next, we wanted to test the cross-protective efficacy of an RVFV strain ZH548-derived Gn+Gc vaccine against Ken06, SA01, and rSA51. Three groups of 10 BALB/c mice were subcutaneously vaccinated with 5ug each Gn+Gc formulated in ISA25 VG oil-in-water adjuvant and boosted with the same dose 21 days later. Mock mice were administered adjuvant and sterile media. ISA25 VG adjuvant was used in this study because of its efficacy in both mice and sheep.[118,128]

Vaccination with Gn+Gc elicited a detectable Gn-specific anamnestic antibody response in all mice on 28dpfv (Fig 3-4). However, Gc-specific antibody titers were nearly undetectable although they were above the cutoff value of 0.04. On day 35, all mice were challenged subcutaneously with x1000 LD_{50} of RVFV strains Ken06, SA01, or rSA51 (Table 2-2) and monitored for survival for 12 days. All vaccinated mice challenged with either Ken06 or SA01 survived until the end of the study, while only 50% of the vaccinated rSA51 challenged mice

survived (Fig 3-5). In contrast, 30%, 10%, and 0% of mock vaccinated mice survived when challenged with Ken06, SA01, and rSA51, respectively (Fig 3-4). Although 100% mortality in all mock vaccinated animals was not achieved, statistically significant differences in survival were seen between vaccinated and mock vaccinated animals. These data suggest that the combined Gn+Gc recombinant antigen derived from ZH548 fully protects against lethal challenge with Ken06 and SA01, and modestly protects against rSA51.

Figure 3-4 Day 21 and 28dpfv antibody responses following vaccination with Gn+Gc

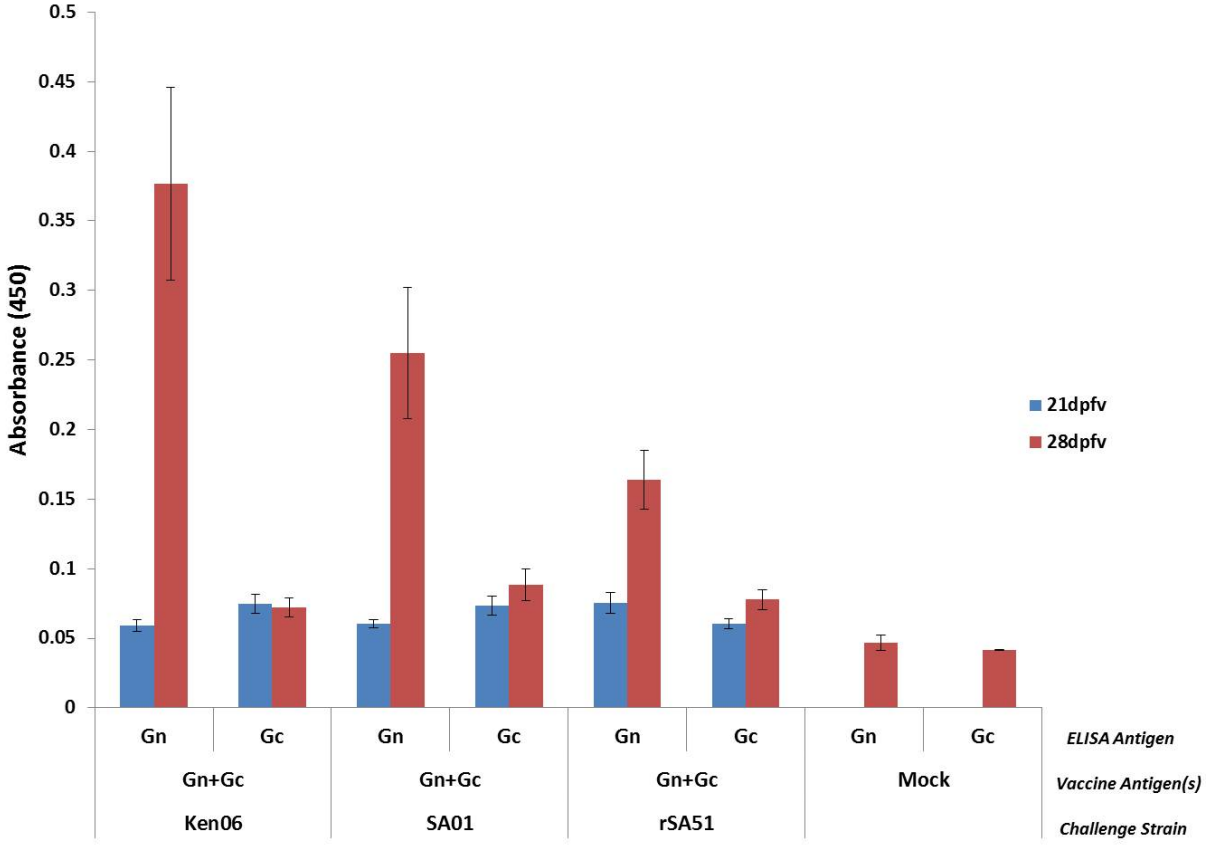

Fig 3-4. Vaccine-induced antigen-specific IgG response to Gn+Gc vaccination. Each bar represents the mean of 10 absorbance of n=10 mouse sera. Gn and Gc mock bars represent mean absorbance of n=3 time-matched mouse sera. The cutoff value for Gn = 0.067 and Gc = 0.0432. Positive control was from a Gn+Gc vaccinated sheep at 28dpfv. Gn positive control absorbance was 2.40 and Gc was 2.55.

Figure 3-5 Surival of Gn+Gc vaccinated mice challenged with 3 RVFV strains

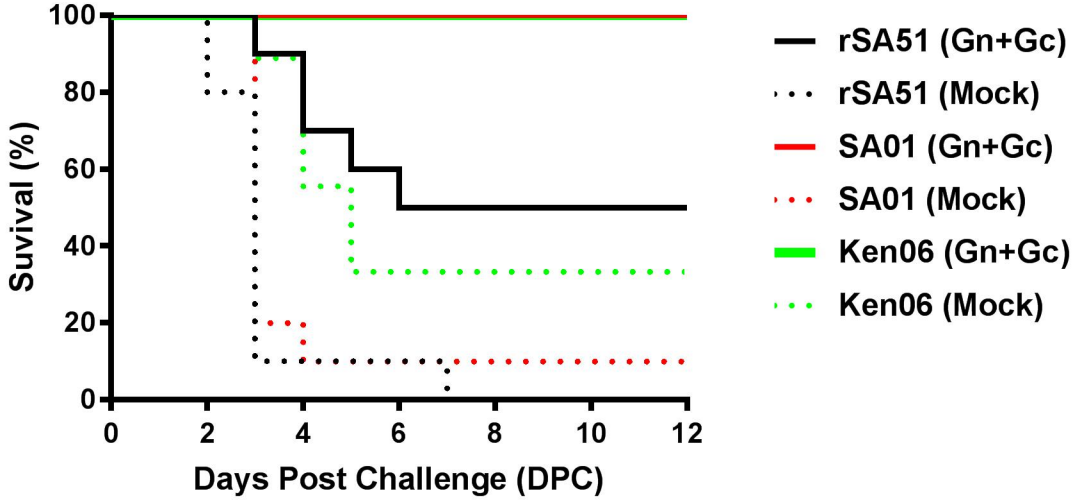

Fig 3-5. Vaccination with recombinant Gn+Gc antigen differentially protects mice against RVFV challenge strains. Groups of 10 mice were vaccinated with 5ug each Gn and Gc formulated in ISAVG adjuvant or mock vaccinated with adjuvant formulated in sterile media. On 35dpfv, 7dpsv mice were challenged with x1000 LD_{50} of RVFV rSA51, SA01, or Ken06 and monitored for survival 3x daily until 12 days post-challenge.

Table 3-7 Comparison of survival rates of vaccinated vs. mock vaccinated mice challenged with different RVFV strains

Challenge Strain	Vaccination	Survival Rate	Statistical Significance (p-value)
Ken06	Gn+Gc	10/10	0.0021
Ken06	Mock	3/10	
SA01	Gn+Gc	10/10	<0.0001
SA01	Mock	1/10	
rSA51	Gn+Gc	5/10	0.0011
rSA51	Mock	0/10	

Table 3-4. Comparison of survival rates of Gn+Gc vaccinated and mock vaccinated mice challenged with different RVFV strains. Survival curves between vaccinated and mock-vaccinated mice from Fig. 3-5 were analyzed via Log Rank test (Mantel-Cox).

3.7 Gn+Gc vaccination reduces virus replication in target tissues following challenge with Ken06 and SA01

To assess the efficacy of Gn+Gc vaccination against heterologous strain challenge in target tissues, we vaccinated and boosted a separate group of mice (n=5/group) with 5ug each Gn and Gc as above. Mock vaccinated mice were given sterile media with adjuvant. As previously, antibody titers to Gn were significantly higher than Gc for both vaccinated groups, though again Gc antibody titers were above the determined cutoff value (Fig. 3-6). On 35dpfv all mice were challenged with x1000 LD_{50} of RVFV strains Ken06 and SA01 and subsequent plaque assays were performed on homogenized tissues from found-dead or culled mice 3dpc (Fig. 3-7). Mean viral titers in liver and spleen were similar in mock vaccinated mice infected with either virus (Ken06 liver = $5.48x10^5$ pfu/g, spleen = $2.14x10^5$ pfu/g; SA01 liver = $1.23x10^6$ pfu/g, spleen = $2.0x10^5$ pfu/g). Mean viral titers in the brains of mock-vaccinated mice, however, were approximately 2 logs greater in Ken06 vs. SA01-infected mice (Ken06 = $2.52.x10^4$ pfu/g, SA01 = 118 pfu/g). Conversely, decreases in mean tissue titers were seen in all tissues for vaccinated mice infected with either virus (Ken06 liver = 0 pfu/g, spleen = 56 pfu/g, brain = 12 pfu/g; SA01 liver = 30 pfu/g, brain = 8 pfu/g). However, none of these differences are statistically significant because of the large variation seen in tissue titers within each group (Table 3-8). Although sterilizing immunity was not achieved in the vaccinated groups, these data further suggest that a ZH548-derived recombinant Gn+Gc vaccine is cross-protective against strains Ken06 and SA01.

Figure 3-6 28dpfv ELISA of mice vaccinated with Gn+Gc

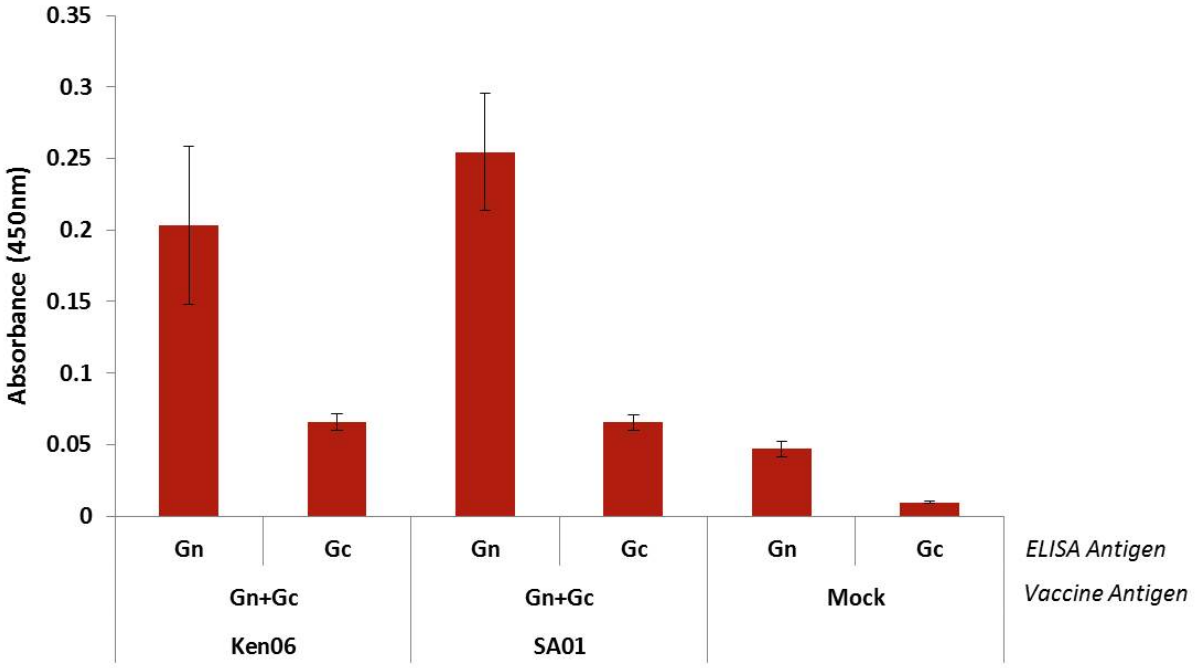

Fig. 3-6. Vaccine-induced antigen-specific IgG response to Gn+Gc vaccination. Each bar represents the mean absorbance of n=5 Gn+Gc vaccinated mouse sera on 28dpfv. Gn and Gc mock bars represent mean absorbance of n=3 time-matched mouse sera. The cutoff value for Gn = 0.067 and Gc = 0.0432. Positive control was from a Gn+Gc vaccinated sheep at 28dpfv. Gn positive control absorbance was 2.69 and Gc was 2.68.

Figure 3-7 3dpc tissue titers from Gn+Gc vaccinated and mock vaccinated mice challenged with Ken06 or SA01

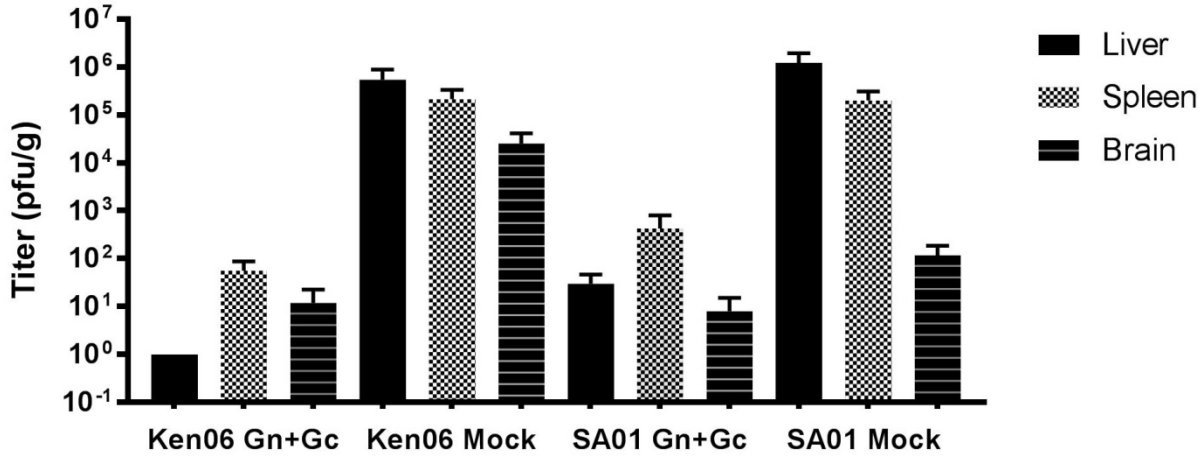

Fig 3-7. Tissue titers of Gn+Gc vaccinated and mock vaccinated mice challenged with Ken06 or SA01 and necropsied on 3dpc. N=5 spleen, liver, and brain homogenates from each group were collected at necropsy on 3dpc and subsequently homogenized. Tissue homogenates were titered via plaque assay. Error bars on each bar represent standard error of the mean.

Table 3-8 Statistical comparisons of virus replication between Gn+Gc vaccinated and mock vaccinated mice

Challenge Strain	Vaccination	Tissue	Virus Titer Range (PFU/g)	Statistical Significance (p-value)
Ken06	Mock	Liver	0 - 2000000	0.093
Ken06	Mock	Spleen	0 - 700000	0.074
Ken06	Mock	Brain	0 - 92000	0.1
SA01	Mock	Liver	0 - 4000000	0.077510326
SA01	Mock	Spleen	0 - 500000	0.071
SA01	Mock	Brain	0 - 410	0.093

Table 3-8. Statistical comparison of vaccinated versus mock vaccinated mice using one-tailed T-test (5th column). Range of tissue titers of mock vaccinated animals in various tissues.

3.8 Gn and Gc differentially protect against homologous RVFV challenge

Since Gn and Gc elicit a protective immune response when administered together, we sought to determine the immunogenicity and protective efficacies Gn and Gc when administered individually. Groups of 10 mice were vaccinated with 10ug Gn or Gc in adjuvant and boosted on day 21 with the same dose. Serum collected on 21 and 28dpfv was analyzed via ELISA and serum collected on 35dpfv was analyzed PRNT80. As before, Gn elicited an appreciable, antigen specific antibody response at 28dpfv (mean OD value = 0.42). However, although Gc did elicit some detectable antibodies above background at 28dpfv (mean OD value =0.08), this response was significantly less compared to Gn.

To compare the relative virus neutralizing efficacies of Gn and Gc antigens, we performed $PRNT_{80}$ assays on 35dpfv serum from mice vaccinated with Gn-only, Gc-only, or Gn+Gc (n=5 per group). Mean reciprocal titers of mice vaccinated with Gn-only was 48, while Gn+Gc and Gc-only vaccinated mice had demonstrated reciprocal titers of 20 and <20, respectively. Pooled, pre-immune sera from 0dpfv mice were used as a negative control demonstrating non-detectable neutralizing titers. These data suggest that Gn induces greater antigen-specific IgG and neutralizing antibody titers than Gn+Gc and Gc-only.

Figure 3-8 Antigen specific serological responses of mice vaccinated with Gn or Gc

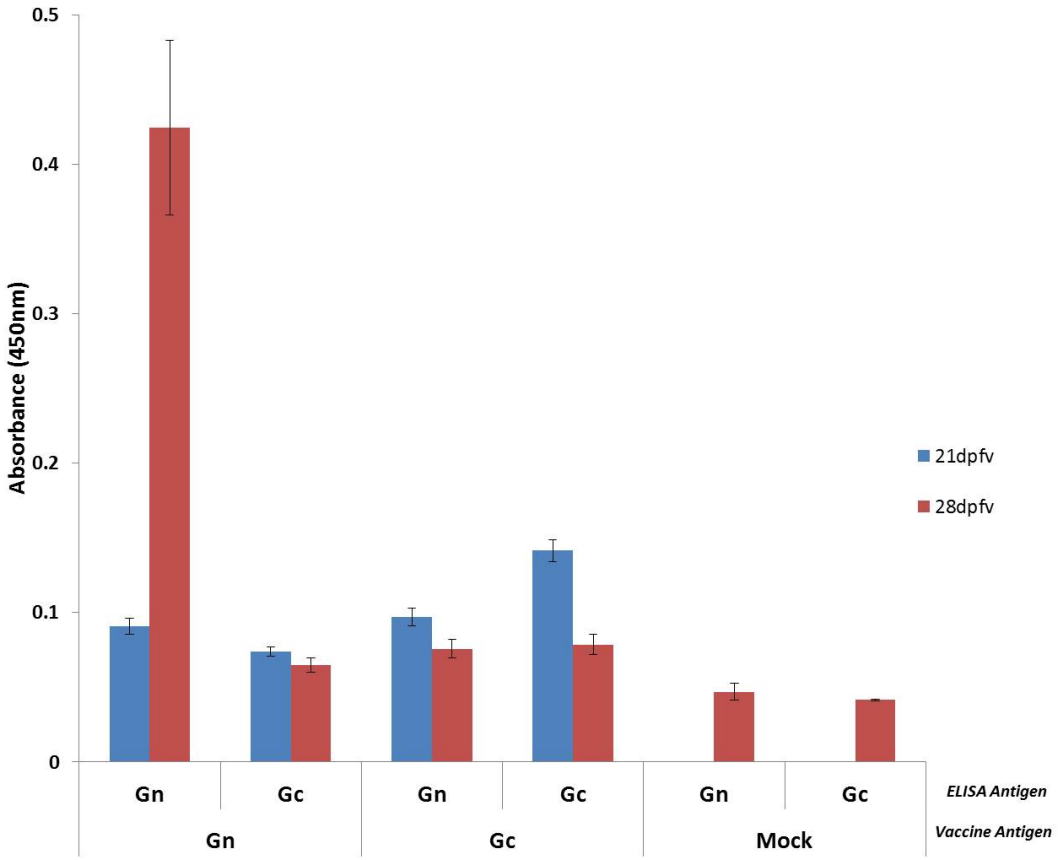

Fig 3-8. Vaccine-induced antigen-specific IgG response to Gn+Gc vaccination. Each bar represents the mean absorbance of n=10 Gn- or Gc-vaccinated mouse sera on 28dpfv. Gn and Gc mock bars represent mean absorbance of n=3 time-matched mouse sera. The cutoff value for Gn = 0.067 and Gc = 0.0432. Positive control was from a Gn+Gc vaccinated sheep at 28dpfv. Gn positive control absorbance was 2.31 and Gc was 2.55.

Table 3-9 $PRNT_{80}$ Titers of Serum from Vaccinated Mice Collected on Challenge Day

Replicate	Vaccine	Reciprocal Titer	Vaccine	Reciprocal Titer	Vaccine	Reciprocal Titer
1	Gn+Gc	20	Gn	80	Gc	<20
2	Gn+Gc	20	Gn	20	Gc	<20
3	Gn+Gc	20	Gn	40	Gc	<20
4	Gn+Gc	20	Gn	80	Gc	<20
5	Gn+Gc	20	Gn	20	Gc	<20
Mean	**Gn+Gc**	**20**	**Gn**	**48**	**Gc**	**<20**

Table 3-9. $PRNT_{80}$ of 35dpfv mice vaccinated with Gn+Gc, Gn-only, or Gc-only.

3.9 Gn and Gc differentially protect against RVFV challenge

On day 35, mice were challenged with x1000 LD_{50} of Ken06 and monitored 12 days for survival. All 10 vaccinated with Gn survived until the end of the study except 1 mouse, which died on day 12. In contrast, only 4/10 Gc vaccinated animals survived until the end of the study (Fig 3). When analyzed via Log Rank test, significant differences in survival time were seen between Gn and mock groups but not between Gc and mock groups. Taken together, this suggests that Gn alone can elicit protective immunity but Gc cannot.

Figure 3-9 Survival of mice vaccinated with Gn-only or Gc-only and challenged with RVFV Ken06

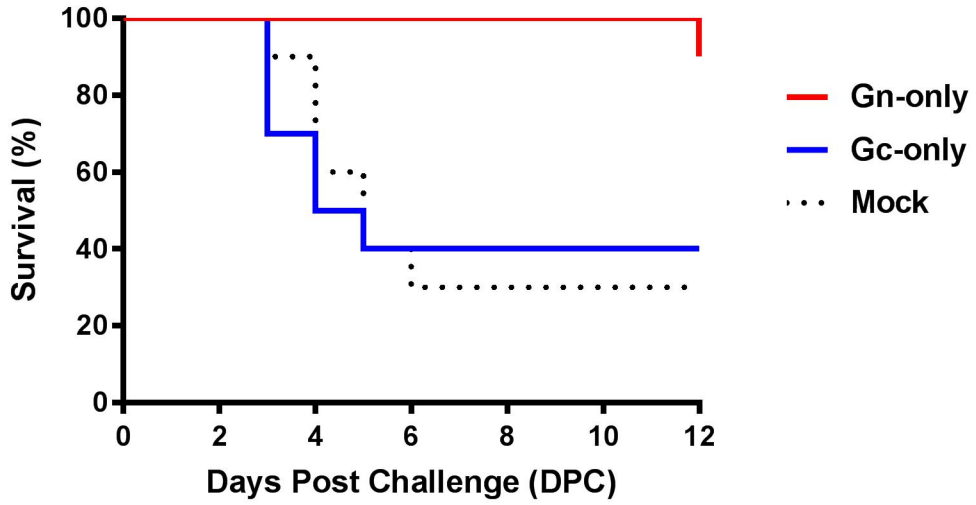

Fig 3-6. Vaccination with Gn but not Gc protects mice against challenge with Ken06. Groups of 10 mice were vaccinated with 5ug either Gn or Gc formulated in ISAVG adjuvant or mock vaccinated with adjuvant formulated in sterile media. On 35dpfv, 7dpsv mice were challenged with x1000 LD_{50} of RVFV rSA51, SA01, or Ken06 and monitored for survival 3x daily until 12 days post-challenge

Chapter 4 - **Discussion and Conclusions**

RVFV is considered a major threat to US agriculture and public health due to its ability for transboundary spread. Therefore, vaccine development remains a high priority since there are currently no fully licensed vaccines outside endemic areas. Though many RVFV vaccine candidates are being developed, vaccines such as glycoprotein subunit vaccines remain a viable candidate due to their efficacy, production scalability, and DIVA compatibility – the latter of which is essential to determining disease eradication. The objectives of this study were to (1) characterize the virulence of 3 genetically distinct RVFV strains Ken06, SA01, and rSA51, and determine the (2) cross-protective and (3) antigen-specific protective efficacies of recombinant RVFV glycoproteins against these three strains.

RVFV strains Ken06, SA01, and rSA51 demonstrated maximum sequence divergences of 0.7, 1.1, and 1.37% across the L, M, and S amino acid sequences, respectively. These divergences are within the limits of those described elsewhere. When we analyzed these strains via one-step growth curves in MRC5 cells, SA01 replicated less efficiently than Ken06 and rSA51. This is an interesting result considering that SA01 NSs amino acid sequence differs from Ken06 and SA51 by 0.92% (11 substitutions), and 1.1% (13 substitutions), respectively, and that NSs is a driver of virus replication and virulence.[124] Therefore, one or more of these mutations could be contributing to SA01 reduced growth in cell culture. However, further work needs to be conducted to fully elucidate this.

In order to quantitate virulence and standardize challenge doses for subsequent vaccine-challenge studies we determined the LD_{50} of 3 distinct strains of RVFV (Ken06, SA01, and rSA51) using a BALB/c mouse model. The LD_{50} values of Ken06 and SA01 were both 7.9pfu, while the LD_{50} of rSA51 was 149.7pfu. These LD_{50}values are comparable to those determined for other virulent wildtype RVFV strains (Darci R. Smith, pers. comm. 2015).[43] However, LD_{50}

has been shown to be mouse strain dependent. For example, previous researchers showed that RVFV strain Ken57 (Rintoul) was highly virulent in C57BL6 mice (LD_{50} = 2pfu) but severely attenuated in ICR mice (LD_{50} = $3.7x10^3$pfu). Additionally, although our data demonstrated an LD_{50} = 149.7 for rSA51 in subcutaneously challenged BALB/c mice, previous researchers calculated an LD_{50} of <1 and 8pfu in intraperitoneally challenged ICR and C57BL/6 mice, respsectively.[43] Interestingly, a wild-derived non-recombinant SA51 isolate was used in their study, suggesting there may be virulence differences between wild-derived and recombinant isolates of the same RVFV strain. In support of this, significant differences in virulence between recombinant and wild-derived SA51 were demonstrated in CD1 mice challenged intraperitoneally (P=0.0181; Ikegami 2015 unpublished). Taken together, these data suggest that mouse breed, challenge route, and virus source can significantly influence LD_{50}.

Previous researchers have suggested that one vaccine will cross-protect against all known strains of RVFV based on the high conservation of immunogenic glycoproteins Gn and Gc.[41] However, this question has not been pursued in animal models or through the analysis of genetic diversity in specific immunogenic epitopes of Gn and Gc. This information is critical to evaluating vaccine efficacy and informing licensure decisions. Within the 3 strains analyzed, our data showed low amino acid sequence diversity within Gn and Gc ectodomains and no divergence within 3 characterized neutralizing epitopes within Gn. Although there was some sequence variation within these epitopes among the strains available on GenBank, no strains had mutations in all 3 neutralizing epitopes. Since monoclonal antibodies specific to these regions have been shown to induce protective immunity *in vivo*, mutations in all 3 epitopes may be required for immune escape.

Gn+Gc vaccinated mice were fully protected against challenge with x1000 LD_{50} of Ken06 and SA01 and partially against rSA51. This expands on our previous data that a ZH548-

derived subunit Gn+Gc vaccine fully protects sheep against challenge with Ken06. The lack of efficient cross-protection against rSA51 may be due to immune escape since rSA51 Gn and Gc ectodomains differ somewhat from that of ZH548. However, Gn+Gc vaccinated mice challenged with rSA51 had lower Gn-specific ELISA titers on 28dpfv than that of Ken06 and SA01-challenged mice. Additionally, although challenge doses were standardized by LD_{50}, rSA51-challenged mice received ~19 times more virus than SA01 or Ken06-challenged mice, which could be greater than the maximum amount of antigen able to be neutralized by available serum neutralizing antibodies. Ideally, to address this concern in future cross-neutralization studies, a wide range of challenge doses should be used.

To bolster our survival data, and to compare virus titers within vaccinated and mock-vaccinated animals we vaccinated separate groups of mice with Gn+Gc and challenged as before with x1000 LD_{50} with Ken06 or SA01. Although sterilizing immunity was not seen, decreases in viral load were seen in all 3 target organs (liver, spleen, brain) in mice vaccinated with Gn+Gc compared to mock vaccinated animals on 3dpi. However, these decreases were not significant due to large variation in titers from mock and vaccinated animals, a clear decreasing trend in virus titers in each tissue was seen. This large variation tissue titers of mock-vaccinated animals may be due to differences replication kinetics within each mouse in the sense that maximum tissue titers may be achieved at different time points in each mouse. Because only one sampling time point was used this possibility cannot be ruled out. Additionally, subcutaneous challenge may be a less efficient route of infection than intraperitoneal.

Monovalent Gn-based vaccines have been shown to be effective in mouse models[57]. However, no studies have directly compared the protective efficacies of different RVFV glycoprotein constructs, i.e. Gn+Gc, Gn-only, Gc-only. Therefore, we vaccinated mice with 3 different RVFV subunit glycoprotein vaccine constructs and assessed their protective efficacies

against challenge with Ken06. RVFV glycoprotein constructs Gn+Gc and Gn-only, offered 100% (10/10) and 90% (9/10) protection against lethal challenge with Ken06, thereby expanding on data from our previous study in which Gn+Gc was able to fully prevent virus replication and disease in sheep[120]. Conversely, vaccination with the Gc-only offered no protection. However, since Gc was shown not to be antigenic or immunogenic via ELISA and $PRNT_{80}$ assay in our study, but has been shown to be antigenic in other studies, we cannot eliminate the possibility that Gc is not a protective immunogen.[120] We plan to re-express and purify recombinant Gc and repeat this study in the future to account for any potential protein stability issues. These data do confirm, however, that Gn alone can induce neutralizing antibodies and protective immunity *in vivo*. This is significant since an efficacious Gn-only vaccine would be cheaper to produce than a bivalent vaccine containing both glycoproteins.

In summary, these data suggest that a Gn-only vaccine is an efficacious alternative to Gn+Gc subunit vaccine constructs and that Gn+Gc vaccines have the potential to cross-protect against all known RVFV strains. These data can be used to optimize current RVFV vaccine strategies, design confirmatory studies in ruminants, and inform vaccine licensure decisions

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