Human vaccine candidate (DDVax) development against Rift Valley Fever: dissemination safety studies in mosquitoes

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Abstract

Rift Valley fever virus (RVFV) is a mosquito-borne pathogen with significant human and veterinary health consequences that periodically emerges in epizootics. RVF causes fetal loss and death in ruminants and in humans can lead to liver and renal disease, delayed-onset encephalitis, retinitis, and in some cases severe hemorrhagic fever. A live attenuated vaccine candidate (DDVax), was developed by the deletion of the virulence factors NSs and NSm from a clinical isolate, ZH501, and has proven safe and immunogenic in rodents, pregnant sheep and non-human primates. Deletion of NSm also severely restricted mosquito midgut infection and inhibited vector-borne transmission. To demonstrate environmental safety, this study investigated the replication, dissemination and transmission efficiency of DDVax in mosquitoes following oral exposure compared to RVFV strains MP-12 and ZH501. Infection and dissemination profiles were also measured in mosquitoes 7 days after feeding on goats inoculated with DDvax or MP-12. Hypothesis: DDVax should infect mosquitoes at significantly lower rates than other RVF strains and, due to lack of NSm, be transmission incompetent. Exposure of *Ae. aegypti* and *Cx. tarsalis* to 6-8 log $_{10}$ plaque forming units (PFU)/mL DDVax by artificial bloodmeal resulted in significantly reduced DDVax infection rates in mosquito bodies compared to controls. Plaque assays indicated negligible transmission of infectious DDVax in *Cx. tarsalis* saliva (1/140 sampled) and none in *Ae aegypti* saliva (0/120). Serum from goats inoculated with DDVax or MP-12 did not harbor detectable infectious virus by plaque assay at 1, 2, or 3 days-post-inoculation; infectious virus was, however, recovered from mosquito bodies that fed on goats vaccinated with MP-12 (13.8% and 4.6%, respectively), but strikingly, DDvax positive mosquito bodies were greatly reduced (4%, and 0%, respectively). Furthermore, DDVax did not disseminate to legs/wings in any of the goat-fed mosquitoes. Collectively, these results are consistent with a beneficial environmental safety profile .

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Summary

Rift Valley fever virus (RVFV) is a mosquito-borne pathogen with significant human and veterinary health consequences that periodically emerges in epizootics. RVF causes fetal loss and death in ruminants and in humans can lead to liver and renal disease, delayed-onset encephalitis, retinitis, and in some cases severe hemorrhagic fever. A live attenuated vaccine candidate (DDVax), was developed by the deletion of the virulence factors NSs and NSm from a clinical isolate, ZH501, and has proven safe and immunogenic in rodents, pregnant sheep and non-human primates. Deletion of NSm also severely restricted mosquito midgut infection and inhibited vector-borne transmission. To demonstrate environmental safety, this study investigated the replication, dissemination and transmission efficiency of DDVax in mosquitoes following oral exposure compared to RVFV strains MP-12 and ZH501. Infection and dissemination profiles were also measured in mosquitoes 7 days after feeding on goats inoculated with DDvax or MP-12. Hypothesis: DDVax should infect mosquitoes at significantly lower rates than other RVF strains and, due to lack of NSm, be transmission incompetent. Exposure of *Ae. aegypti* and *Cx. tarsalis* to 6-8 $\log_{10}$ plaque forming units (PFU)/mL DDVax by artificial bloodmeal resulted in significantly reduced DDVax infection rates in mosquito bodies compared to controls. Plaque assays indicated negligible transmission of infectious DDVax in *Cx. tarsalis*saliva (1/140 sampled) and none in *Ae aegypti* saliva (0/120). Serum from goats inoculated with DDVax or MP-12 did not harbor detectable infectious virus by plaque assay at 1, 2, or 3 days-post-inoculation; infectious virus was, however, recovered from mosquito bodies that fed on goats vaccinated with MP-12 (13.8% and 4.6%, respectively), but strikingly, DDvax positive mosquito bodies were greatly reduced (4%, and 0%, respectively). Furthermore, DDVax did not disseminate to legs/wings in any of the goat-fed mosquitoes. Collectively, these results are consistent with a beneficial environmental safety profile.

1 Introduction

Rift Valley fever virus (RVFV) (family *Phenuiviridae* , genus*Phlebovirus*) is a mosquito-borne virus that causes periodic epizootic outbreaks across Africa and the Arabian peninsula(Al-Afaleq & Hussein, 2011; Nguku et al., 2010). In ruminants, primarily sheep, goats, camels and camelids, RVF is often characterized

by sudden epizootics marked by near universal fetal death at all stages of gestation, congenital malformations(Coetzer, 1982) and significant adult animal deaths often due to acute virus induced hepatic and renal pathology(Wichgers Schreur et al., 2021). Though most human cases are typically self-limiting with mild to moderate symptoms (McElroy, Harmon, Flietstra, Nichol, & Spiropoulou, 2018), cases of delayed onset encephalitis, kidney and/or eye damage, severe anemia, hemorrhagic fever and miscarriage can occur(Baudin et al., 2016; Coetzer, 1982; Madani et al., 2003; Oymans, Wichgers Schreur, van Keulen, Kant, & Kortekaas, 2020).

Over 40 species of mosquitoes have been demonstrated as competent vectors for RVFV(reviewed in(Lumley et al., 2017)), some of which range on multiple continents. Following periods of heavy rainfall, which stimulate rapid increases in vector mosquito populations, RVFV re-emerges periodically in explosive epizootics(Al-Afaleq & Hussein, 2011; Nguku et al., 2010). In the absence of humans and livestock, RVFV cycles between mosquitoes and wild ruminants(Britch et al., 2013; Clark, Warimwe, Di Nardo, Lyons, & Gubbins, 2018). Between epizootics, there is also support for low level maintenance of RVFV in livestock in inter-epidemic periods(Lichoti et al., 2014).

Due to these health implications and the potential to cause a public health emergency, in 2018 the World Health Organization listed RVFV as a research and development blueprint priority pathogen(Mehand, Al-Shorbaji, Millett, & Murgue, 2018). Availability of a safe and effective human vaccine against RVFV is essential to protect the health of people in endemic regions and a preparatory measure for the anticipated cross-border spread and establishment of RVFV into new geographic areas. A number of vaccine candidates have been developed for RVFV, including formalin inactivated (Pittman et al., 1999; Randall, Gibbs, Aulisio, Binn, & Harrison, 1962) and live attenuated strains(Ikegami et al., 2015; Smithburn, 1949). However residual teratogenic effects in animals or the need for boosters to maintain protective immunity(Bird, Ksiazek, Nichol, & Maclachlan, 2009; Botros et al., 2006) present challenges for further development of these earlier candidates. To date, there is currently no commercially available and fully FDA-approved RVFV human vaccine.

To meet this critical health need, a human vaccine candidate (DDVax), a double deletion construct of the parental wild-type strain ZH501 was generated using a reverse genetics approach wherein both the NSs (non-structural, S segment) and NSm (non-structural, M segment) virulence genes were removed(Bird et al., 2008). NSs is expressed in an ambisense fashion from the viral S segment(Ikegami et al., 2009) and is a multi-functional protein that antagonizes host cell interferon responses(Le May et al., 2008). The viral M segment encodes 2 major glycoproteins and multiple open reading frames in the NSm coding regions, which is required for efficient dissemination in mosquitoes(Crabtree et al., 2012). Neither NSs nor NSm are required for viral replication in interferon-deficient cell culture, and the attenuated DDVax vaccine candidate has proven to be safe and immunogenic in a variety of animal species with the added benefit of inhibited replication and transmission in mosquitoes(Bird et al., 2008; Bird et al., 2011; Crabtree et al., 2012; Kading et al., 2014).

The objective of this study was to confirm that the newly rescued version of DDVax produced for development under Good Manufacturing Practices (GMP) behaved as previously described and exhibited a highly favorable environmental safety profile by not being transmitted by potential mosquito vectors. Here, we describe characterization of RVFV DDVax in mosquitoes using both *in vitro* and *in vivo*approaches. These vector assessments were divided into two experimental phases: 1) mosquito oral challenges via artificial feeding and 2) mosquito feeding on DDVax inoculated goats. Features of vector competence were measured in two competent mosquito species, *Culex tarsalis* Coquillett and *Aedes aegypti* L., to determine infection, dissemination and transmission potential, using reverse transcriptase- quantitative PCR (RT-qPCR) and infectious virus plaque assay. Vertebrate-to-vector transmission from DDVax-inoculated goats to mosquitoes was also measured. Collectively, these experiments provided important comparison of vector competence of mosquitoes exposed to DDVax(Bird et al., 2008), ZH501, the parental wild-type virus and MP-12, an existing vaccine virus strain(Turell & Rossi, 1991).

2 Methods

2.1 DDVax Production Process Summary

2.1.1 Uninfected Cell Culture

Serum-Free Vero cells (2 x 10^4cells/cm^2) were grown in OptiPro Serum-Free Media (SFM) (Lifetech, A31343) with Glutamax (Gibco, 35050-061) at 37°C and 5% CO_2. Cells were expanded into 3 x 10-Layer CellStacks (6360 cm^2) (Corning, 3271) and 1 x 1-Layer CellStacks (636 cm^2) (Corning, 3268). For each passage, cells were seeded at either 2.0 x 10^4cells/cm^2 for 48 ± 8-hours or 1.5 x 10^4 cell/cm^2 for 72 ± 8-hours. Cell harvest was performed using TrypLE Select (Life Tech, 12563092). Cells were centrifuged at 500 x g for 5 minutes at 18°C and resuspended in OptiPro SFM. Cell enumeration was performed using a Vi-Cell Cell Viability Analyzer.

2.1.2 Generation of DDVax Pilot Material

Upon achieving cell confluency within the 3 x 10-Layer or 1 x 1-Layer CellStacks, Vero cells were infected with the DDVax Research Virus Stock (RVS), Lot # N16-5-20-RV at a multiplicity of infection (MOI) of 0.0005 PFU/cell. Infection volume used for each 10-Layer CellStack was 1300ml and the infection volume for the 1-Layer CellStack was 130ml. Infected cultures were then incubated at 37°C and 5% CO_2 for 72-hours. Following the 72-hour infection incubation, flasks were examined for cytopathic effects (CPE). DDVax virus was then harvested by pumping the supernatant from each CellStack into a 5L Flexboy bag. The harvested pool underwent Benzonase treatment to digest Host Cell DNA (HCD). A 500mM $MgCl_2$ solution was added to the Flexboy bag to achieve a final concentration of 1.5mM $MgCl_2$ for Benzonase treatment. Benzonase (EMD Millipore, 101679) was added at a concentration of 50,000 U/L. The Flexboy bag was thoroughly rocked to mix and then incubated for 60 minutes at 37°C and 5% CO_2, with rocking at 10-minute intervals. The Benzonase-treated pool was divided into 500ml conical tubes and centrifuged at 3000 x g for 15 minutes at 18°C to remove large cell debris. Supernatant containing the DDVax, now the clarified pool, was pumped out of the centrifuge bottles into a new 5L Flexboy bag. Virus was then concentrated by a factor of 6 using ultrafiltration (UF) using the KMPi TFF system (Repligen) over a 500kD Hollow Fiber Membrane (Repligen, S02-E500-05-N, 500kD, 20cm length, 0.5mm fiber diameter). Concentration was performed at a target shear rate of 3000 s^{-1} and TMP setpoint of 5 psi. The concentrated UF pool was diafiltered (DF) into a buffer containing 0.2M NaCl, 10mM Sodium Phosphate, 4% Sucrose, 5mM Glutamax, pH 7.4 ± 0.1. Buffer exchange was performed for 10 diavolumes. Diafiltration was performed at a target shear rate of 3000 s^{-1}

2.2 DDVax sequencing and analysis

Illumina shotgun sequencing libraries were prepared from total RNA using the Kapa RNA HyperPrep kit following the manufacturer's protocol. Dual indexed libraries were sequenced on an Illumina NextSeq 500 sequencer to generate single-end 150 nt reads.

We used two complementary approaches to detect and quantify viral variants. First, we used the lofreq tool to identify single nucleotide variants and short insertions and deletions (Wilm et al., 2012). Second, we used DI-tector to identify structural variants including longer deletions and insertions and copy back defective viral genomes (DVGs)(Beauclair et al., 2018; Vignuzzi & Lopez, 2019). These tools were run as part of a reproducible Nextflow pipeline, available at https://github.com/stenglein-lab/viral_variant_caller/releases/tag/DDVax_paper_release. Software dependencies and reference sequences (DDVax) are captured in this version-controlled release and in the conda environment contained therein.

To quantify variants, adapter-derived and low-quality bases were trimmed using Cutadapt(Martin, 2011). Host cell-derived reads were removed using bowtie2 to align reads to the *Chlorocebus sabeus* genome, accession GCF_000409795.2(Langmead & Salzberg, 2012). Host- and quality-filtered reads were aligned to the S, M,

and L segment RVFV/DDVax reference sequences using the BWA aligner(Langmead & Salzberg, 2012; Li & Durbin, 2009). The reference sequences consisted of the RVFV-derived portions of the DDVax plasmid sequences. To improve accuracy of structural variant (indel) calls, base quality scores were recalibrated using GATK (McKenna et al., 2010). Single nucleotide variants (SNVs) and structural variants were called using LoFreq(Wilm et al., 2012). The minimum depth of coverage to call a variant was set at 40x coverage. SnpEff and SnpSift were used to predict the functional impact of variants(Cingolani, Patel, et al., 2012; Cingolani, Platts, et al., 2012). Variant calling distinguished between variants that were not detected despite sufficient data and positions that lacked sufficient data to call variants. Defective viral genomes were identified using the DI-tector tool(Beauclair et al., 2018). Outputs of these analyses were tabulated, processed, and visualized in R. Variants with frequencies [?] 3% were reported(Grubaugh et al., 2019).

2.3 Virus strains

Stocks of DDVax were produced as described above. The passage history of MP-12 strain is unknown, but DQ375404.1 (L segment), DQ380208.1 (M segment) and DQ380154.1 (S segment) sequences were confirmed by Sanger sequencing. ZH501 strain virus was obtained from R. Bowen. V1 (Vero) passage stock was passaged twice in Vero cells to obtain V3 stocks used for this study.

2.4 Insect cell culture virus growth curves

The insect cell lines used for this study were Ct cells, derived from *Cx. tarsalis* embryos (Centers for Disease Control and Prevention)(Chao & Ball, 1976), Aag2 *Ae. aegypti* high passage cells, also derived from embryos(Chao & Ball, 1976), and ATC10 (CCL-125 (ATCC)), a larval-derived cell line(Singh, 1971). Virus strains RVFV-ZH501, the parental wild-type to DDVax(Bird et al., 2008), MP-12(Turell & Rossi, 1991) and DDVax were subjected to growth curves in mosquito cell culture (ATC-10, Aag2, CT) using Schneider's media (10% FBS (or 20% FBS for ATC-10s), 1% non-essential amino acids, 1% L-glutamine, 1% penicillin/streptomycin). An MOI of 0.01 was used for all infections. Aliquots were removed at daily timepoints for 1-6 days post-infection (dpi). At each timepoint, 400 µl cell culture supernatant was removed, and media was replaced into the T-flask. Aliquots were supplemented with 20% FBS as a cryoprotectant and stored at -80°C until titrations were performed.

2.5 Mosquitoes

The Poza Rica *Ae. aegypti* strain was colonized in 2012 from collections in the state of Veracruz, Mexico(Vera-Maloof, Saavedra-Rodriguez, Elizondo-Quiroga, Lozano-Fuentes, & Black IV, 2015). The *Cx. tarsalis* Kern National Wildlife Refuge (KNWR) colony(Oviedo, Romoser, James, Mahmood, & Reisen, 2011), established in 1952, was obtained from the Centers for Disease Control and Prevention (Fort Collins, CO). Mosquito colonies were maintained at 24-26°C (*Culex*) or 28°C (*Aedes*) at 12:12 light:dark cycle; adults were fed water and sucrose *ad libitum* . Larvae were reared on ground TetraMin fish food (http://www.tetra-fish.com/).

2.6 Vector competence

Adult mosquitoes (4-10 days old) were provided an oral, artificial meal containing freshly grown RVFV. To approximate titers of 7 $\log_{10}$ PFU/ml, virus was harvested 3 days after infection of Vero cells at an MOI of 0.01. Viral supernatant was mixed 1:1 in defibrinated calf blood, with the addition of 1mM ATP, and 0.075% sodium bicarbonate. Mosquitoes were fed for 1 to 1 $\frac{1}{2}$ hours using either a water-jacketed feeder (https://lillieglassblowers.com) for DDVax and MP-12 or a hemotek (http://hemotek.co.uk/), in the case of ZH501. All ZH501 feedings and mosquito incubation steps were performed in the animal biosafety level 3 conditions. All other *in vitro*mosquito oral exposures were conducted in standard biosafety level 3 containment. Fully-engorged mosquitoes were separated into cartons and provided sucrose and water *ad libitum* . Mosquitoes were held for 14 days at ˜80% humidity and 28°C. Infectious blood meal titers were determined through back titration of the infecting blood meals.

At 14 days post-challenge, mosquitoes were anesthetized at 4°C, then held on ice during processing. Tissue samples were dissected, then placed in separate tubes of 250 µl mosquito diluent (DMEM, 20% heat-

inactivated FBS, 50 µg/ml Pen-Strep, 50 µg/ml gentamicin, and 2.5 µg/ml amphotericin B), as follows: Legs and wings were removed for determination of viral dissemination. Saliva was collected for determination of transmission potential. The mosquito proboscis was placed in a capillary tube containing type B immersion oil (Bioworld, SKU- 21750002) and allowed to salivate for 30-60 minutes. At that time, the capillary tube was removed and placed in a tube containing mosquito diluent (1x PBS supplemented

with 20% FBS (heat-inactivated), 50 µg/ml Penicillin/Streptomycin, 50 µ/ml Gentamycin,

2.5µg/ml Fungizone) and centrifuged at 14,000 x g for 3 minutes. Lastly, each remaining body was also placed in a separate tube, for measurement of infection status. Samples were homogenized on a Qiagen Tissuelyzer (Qiagen) at 30 beats per second frequency for 30 seconds, then pelleted at 14,000 x g in a microfuge at 4°C for 3 minutes. Samples were stored in -80°C.

2.7 RNA extractions

RNA extractions of individual 50µl sample aliquots were performed using the Applied Biosystems MagMax-96 Viral RNA extraction kit (AMB1836-5, Thermofisher) following the manufacturer's protocol for manual extraction methods (MAN0017826). Linear polyacrylamide was used as a carrier in place of carrier RNA. Reactions were eluted into 50µl elution buffer and stored in 96-well plates at -80°C.

2.8 Reverse transcription quantitative PCR (RT-qPCR)

RT-qPCR was performed in duplicate using 5 µl sample or RNA standards and run on a QuantStudio 2.0 qPCR platform (Applied Biosystems). Calculated virus amounts were adjusted to account for RNA copy number per tissue. The following primers were used to quantitate RVFV RNA in all samples: RVFL-2912fwdgg TGAAAATTCCTGAGACACATGG, RVFL-2971revAC ACTTCCTTGCATCATCTGATG, RVFL-2950-Probe (FAM)-CAATGTAAGGGGCCTGTGTGGACTTGTG-(BHQ1)(Bird, Bawiec, Ksiazek, Shoemaker, & Nichol, 2007). TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems) was used with final primer concentrations of 500nM and a probe concentration of 100nM. Samples and standards were loaded into 96-well plates and run using fast cycling mode on an AB QuantStudio machine, using the manufacturer's recommended settings. The cycling conditions were as follows: 50°C 5 min (1 cyc), 95°C 20 sec (1 cyc), (95°C 3 sec, 60°C 30 sec (40 cyc)).

RNA copy number standards were developed by amplifying a portion of the L segment from 20 ng plasmid bearing the full length gene(Bird et al., 2008). The RVFL2173_T7_F amplification forward primer (TAATACGACTCACTATAGGGCAGGTGAGCCCTTCATTCT) contained a T7 promoter; RVFL3542_R was the reverse primer (GAGGGGTAAATGGCAAGGTACA). 100 ng input of PCR product was used *in vitro* transcription reactions that incubated for 5 hours at 37°C using the manufacturer's recommendations. Transcription products were stored in 5 µl aliquots at -80°C; they were quantitated using a Qubit fluorometer (ThermoFisher) using the manufacturer's recommendations. For RT-qPCR, fresh aliquots of *in vitro* transcription reactions were serially diluted in 10-fold increments to generate standard curves to relate copy number to raw cycle threshold (Ct value). One standards plate was run for all samples screened on a given day. A representative standard curve was y= -3.3111x + 36.655 R^2= 0.9976, where y= Ct value and x= $\log_{10}$ RNA copy number.

2.9 DDVax dose response experiment

A dose response experiment was performed as a follow up to the mosquito vector competence challenges, which were administered with only a single high titer of over 8.0 $\log_{10}$ PFU/ml. The purpose of this experiment was to test the hypothesis that *Cx. tarsalis* DDVax infection rates vary as a function of virus titer in the artificial blood meal. *Cx tarsalis* were exposed to oral bloodmeals at 6.2, 4.5, or 3.5 $\log_{10}$ PFU/ml and held for 14 days at 28°C, rH 80%. At 14 days-post-feeding legs/wings, saliva and bodies were harvested into mosquito diluent as above in individual tubes and stored at -80°C. Sample processing was performed as described above.

2.10 Goat virus inoculations and mosquito challenge

Mature female, non-pregnant dairy goats of multiple breeds were acquired from a commercial dairy and housed in an Animal Bio-Safety Level 3 facility for the duration of the experiment. Goats were inoculated with 5.6 $\log_{10}$ PFU freshly grown MP-12 or 6.6 $\log_{10}$ PFU DDVax, as determined by plaque assay. Blood was drawn from goat jugular vein at days 1, 2, and 3 post-inoculation into gel serum separator tubes (Becton Dickson, https://www.bd.com/); serum was collected by spinning at 1200 *x g* for 10 minutes. Serum was aliquoted and stored at -80°C. Serum samples were titered by plaque assay, and RNA was extracted for detection and quantification of viral RNA.

For mosquito feeding, goats were manually restrained, and mosquitoes were held in cartons with mesh bottoms against patches of clipped fur and held for about 30 minutes to allow feeding on days 1 and 2 post-inoculation (Figure S1). Since *Cx. tarsalis* mosquitoes did not feed well on goats, on day 3 post-inoculated, mosquitoes were exposed in the laboratory to freshly-collected goat blood (collected into EDTA tubes (Becton Dickson,*https://www.bd.com/)*) using a water jacketed feeding apparatus heated to 37°C. Engorged mosquitoes were held for 7 days at 28°C, rH 80%. At 7 days-post-feeding, bodies and legs/wings were placed in individual tubes containing mosquito diluent (see above). Samples were homogenized on a Qiagen Tissuelyzer (Qiagen) at 30 beats per second frequency for 30 seconds, then pelleted at 14,000 *x g* in a microfuge at 4°C for 3 minutes. Tubes were stored in -80°C. Infectious virus (CPE+/-) was measured by plaque assay using 100 µl undiluted sample in duplicate to determine the frequency of mosquito bodies bearing infectious DDVax virus or MP12 RVFV (control). For those with RVFV-positive bodies, legs/wings were also titrated by plaque assay to determine the frequency of mosquitoes with disseminated infectious virus.

2.11 Virus titrations

Vero cells were grown to > 95% confluency in Dulbecco's modified eagle media DMEM (5% fetal bovine serum (Atlas Biologicals), 1% sodium bicarbonate, 1% non-essential amino acids, no phenol red) in 6 or 12-well plates. Ten-fold serial dilutions of virus stocks and blood meal aliquots in media were performed. Mosquito samples were used undiluted. *In vitro* challenged mosquito samples had already undergone one freeze-thaw cycle prior to infectious virus detection. For each dilution or sample, one hundred microliters of sample was added to wells, then incubated with rocking for 1 hour, followed by an overlay (0.4% agarose (Lonza Rockland) in DMEM). At 2 days post-infection, overlays (0.33% neutral red (Sigma N2889), 2% agarose in supplemented DMEM) were applied. Plates were read after 24 hours. Ambiguous plaques were more closely examined under an inverted microscope at 40X magnification to better confirm CPE.

2.12 Data Analysis

P was determined by calculating the proportion of viral RNA-positive mosquito bodies for the combined total number of mosquito RNA samples. Dissemination was determined by calculating the proportion of legs/wings RNA samples with detectable RVFV RNA against the total number of mosquitoes exposed. Transmission was determined by calculating the proportion of saliva RNA samples that were RVFV-RNA positive against the total number of mosquitoes exposed. Percent of saliva expectorants containing infectious virus were also calculated by determining the proportion of saliva samples producing detectable CPE by plaque assay among the total number of individuals tested. The percentage of RVFV-infected mosquitoes after feeding on inoculated goats were determined by calculating plaque positive mosquito bodies per total number of mosquitoes assayed. RVFV growth curve titers were analyzed by calculating the highest dilution containing countable plaques and multiplying that by the dilution factor to obtain $\log_{10}$PFU/ml.

All graphing and statistical tests were performed in Prism Graphpad (version 8, https://www.graphpad.com/). χ^2 contingency tests were used to calculate dissemination and transmission rates. Two way ANOVA (analysis of variance) with Geisser-Greenhouse correction was used to determine differences in viral growth kinetics. One way ANOVA was used to determine differences in bloodmeal titers.

3 Results

3.1 DDVax variant analysis

We used sequencing to track the genetic stability of DDVax over 5 passages in Vero cell culture (P1 through P5, MOI 0.0005). The P5 preparation was used for goat inoculations. Total RNA from virus preparations (supernatant: P1-P4, or filtered supernatant: P5) was converted into shotgun Illumina libraries and sequenced on an Illumina NextSeq 500 instrument to produce a median of 1.2x10^{7}single end 150 nucleotide (nt) reads per sample. After removing low quality and adapter-derived bases, a median of 1.1x10^{7}reads (88%) remained per sample. After removing reads mapping to the host cell genome (*Chlorocebus sabeus*), a median of 3.4x10^6 reads (28%) remained. A median of 4.4x10^5 reads mapped to the DDVax reference sequence, producing a median coverage of 6055x across all viral segments (Figure S2). SNVs and short insertion and deletions were quantified using lofreq, and larger structural variants, including possible DVGs, were quantified using DI-tector (Beauclair et al., 2018; Wilm et al., 2012).

The virus remained generally stable over passage in Vero cells. We report variants with [?] 3% frequency in coding sequences (Table 1). Three single nucleotide variants in the glycoprotein precursor gene rose to above 50% frequency by P5 (Table 1). A variant at position 31 (predicted to produce the amino acid change G3E in the DDVax NSm-deleted glycoprotein, equivalent to Gly 133 in the RVFV NSm/Gn/Gc polyprotein, NC_014396) rose to 54% frequency by P5. A variant at position 499 (G159D, equivalent to Gly 289 in ZH501) rose to 55% by P5. And a variant at position 926 (N301K, equivalent to Asn 431 in ZH501) rose to 90% frequency by P5.The highest frequency L segment variant was a synonymous variant at position 4665 that rose to 16% by P5. No variants on the S segment rose above 3% frequency in any sample. Lofreq did not identify any short insertion or deletion variants above 3%. Similarly, DI-tector did not identify any structural variants (larger insertions, deletions, incomplete transcripts consistent with DVGs or copy-back variants) with a frequency [?] 3%.

3.2 Mosquito vector competence

To measure differences in viral infection kinetics, *Ae. aegypti*and *Cx. tarsalis* were challenged with 1:1 mixtures of blood and freshly grown DDVax and then compared against those infected with MP-12 or the ZH501 parental strain. Because of the need to use freshly-grown virus for infections, it was not possible to control for differences in bloodmeal titers. Mean bloodmeal titers ranged from ˜8.1 logs/ml with DDVax to 6.5 or 6.8 $\log_{10}$ PFU/ml in MP-12 and ZH501, respectively (Figure 1A). Thus, DDVax titers were significantly higher than that of the other two virus strains (ANOVA, p=1.8e-5). Virus infection phenotypes were measured by detection of viral RNA in *Cx. tarsalis* bodies, legs/wings and saliva at 14 days post-infection (Figure 1B, Table S1). All saliva samples were also subjected to plaque assay for detection of infectious virus.

The percentage of *Culex* mosquito DDVax viral RNA positive bodies was not statistically different from MP-12 or ZH501 infections (Figure 1B, Table S1). However, the RNA genome copy number in *Culex* or*Aedes* infected with DDVax was at least two $\log_{10}$values lower than those infected with either MP-12 or ZH501 strains, despite exposure of mosquitoes to a DDVax titer over one $\log_{10}$ PFU greater than controls (Figure 2). Dissemination of DDVax viral RNA to *Culex* legs/wings was also significantly reduced compared to MP-12 (χ^2 test,p = 2.078e-07). Moreover, infectious DDVax was detected in only one of 140 *Culex* saliva samples at 14 dpi, whereas 96% and 82% of MP-12 and ZH501 infected saliva samples, respectively, showed CPE consistent with the presence of infectious virus (Table 2, χ^2 test, p = 2.2e-16 vs MP-12, 2.2e-16, vs ZH501). To rule out the possibility that sample freeze-thaw compromised virus viability, an additional subset of saliva samples from 14 dpi DDVax exposed mosquitoes were assessed for the presence of infectious virus; still, none was detected (Table 2).

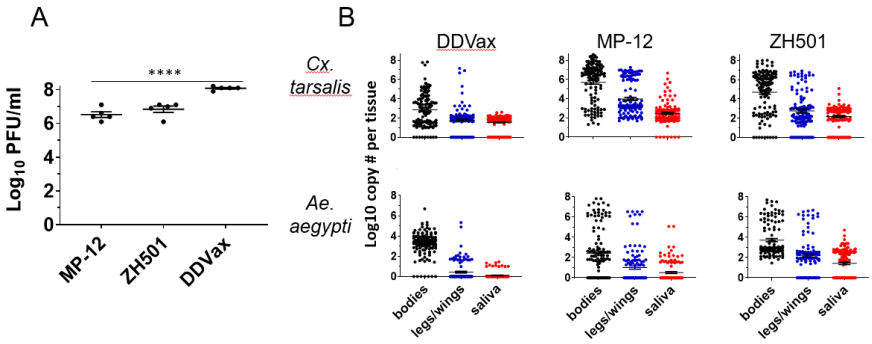

Figure 1. Viral RNA detection in RVFV DDVax, MP-12 and ZH501*in vitro* challenged mosquito bodies, legs/wings and salivary expectorants at 14 dpi. Sample positivity rates are listed in S1 Table. Viral copy number was calculated using a standard curve of diluted L segment transcripts amplified from a plasmid. Profiles from 3 biological replicates were combined, with approximately 40 mosquitoes per replicate.

Ae. aegypti from the *in vitro* virus exposure experiments also showed significantly reduced dissemination in DDVax-infected mosquitoes compared to those challenged with MP-12 or ZH501, respectively (χ^2 test, vs MP-12 $p = 0.02$, vs ZH501 $p =$ 2.2e-16), as indicated by the presence of viral RNA in legs/wings (Figure 1B). *Aedes aegypti* mosquitoes exposed to DDVax had no evidence of infectious virus in expectorated saliva, whereas 16% and 27% of saliva samples were CPE-positive in MP-12 and ZH501 infected mosquitoes, respectively (Table 2, χ^2 test, vs MP-12$p =$ 2.2e-16, vs ZH501 2.821e-09).

3.3 Dose response curve

We expected that DDVax would not be found at significant levels outside mosquito midguts, as described in previous reports of plaque assays for infectious virus(Crabtree et al., 2012). However, our challenge experiments showed unexpectedly high levels of DDVax RNA-positive, CPE-negative saliva samples (Table 2, Table S1, Figure 1B). We hypothesized that the high levels of DDVax viral RNA in saliva may have been due to the high viral titer of the infectious bloodmeal (Figure 1A), which could have overwhelmed natural infection barriers. Therefore, to confirm that viral RNA positivity varied as a function of bloodmeal titer, a second DDVax challenge was performed with *Cx. tarsalis*mosquitoes, using virus serial dilutions. Bloodmeals containing 6.2, 4.5, and 3.5 $\log_{10}$ PFU/ml DDVax were provided. There was a trend for reduction of viral RNA in bodies, legs/wings, and saliva samples as the bloodmeal titer decreased (Table S2, Figure S3). However, strikingly, there was still detectable viral RNA in salivary expectorants with all viral dilutions, including the 3.5 $\log_{10}$ PFU/ml virus meal.

3.4 Mosquito challenge on inoculated goats

To further test the environmental safety profile of DDVax, goats were inoculated with either DDVax or MP-12 viruses. Mosquitoes were allowed to directly feed on the goats at 1 and 2 days post-inoculation (Figure S1). On day 3, blood was collected into EDTA-tubes and transferred to water-jacketed feeders for mosquito challenge in the laboratory. Numbers of engorged mosquitoes from each daily goat feeding are listed in Table S3. Sera from all goat blood specimens were negative for DDVax or MP-12 by plaque assay at 1, 2, and 3 dpi (limit of detection 1 $\log_{10}$ PFU/ml). However, trace levels of viral RNA were detectable by RT-qPCR (Figure S4). After a 7-day extrinsic incubation period, *Aedes* and *Culex* bodies showed evidence of infectious MP-12 by plaque assay (Figure 2), indicative of midgut infections, as previously described(Crabtree et al., 2012; Kading et al., 2014). Viral prevalence was highest in *Aedes* (28%) exposed to goats at 1 day post vaccination with MP-12 strain; these *Aedes*mosquito infection rates decreased to 12% and 6% in mosquitoes that fed on goats 2 and 3 days post-vaccination, respectively. In contrast, 6% (day 1), 2% (day 2) and 5% (day 3) of *Aedes* mosquitoes that fed on DDVax-inoculated goats were positive for infectious

virus by CPE assay after a 7-day incubation period. Across the time series, *Aedes* mosquitoes exposed to MP-12 vaccinated goats showed significantly higher rates of virus-positive bodies than those exposed goats inoculated with DDVax (χ^2 test, $p = 0.011$). *Culex* showed low rates of MP-12 virus infection ($< 10\%$) and no evidence of infection with DDVax. Four of 87 *Culex* mosquitoes that fed on goats vaccinated with MP-12, and 0/59 *Culex* mosquitoes that fed on goats inoculated with DDVax, showed evidence of infection after a 7-day incubation. The differences in *Culex* were not significant, possibly due to low feeding rates (Table S3). All mosquito bodies that were CPE-positive were assessed for the presence of disseminated live virus in legs/wings. However, none of the mosquitoes that became infected after feeding on inoculated goats showed evidence of infectious virus in disseminated infection (positive legs/wings).

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Figure 2. Infectious DDVax or MP-12 detected in bodies from mosquitoes fed on inoculated goats . *Aedes* or *Culex*mosquitoes were fed on goats (n= 3 per virus strain) as indicated, held for 7 days, then processed for by plaque assay(Table S3). Graph shows percentage of samples at each day post-inoculation that were CPE positive, indicative of infectious virus. No infectious virus was detected in legs/wings samples from virus-positive mosquitoes. *Aedes* DDVax, n= 50, 50 and 98 for days 1, 2, 3 respectively. *Aedes* MP-12, n= 64, 60 and 100 for days 1, 2, 3 respectively. *Culex* DDVax, n= 11, 8 and 40 for days 1, 2, and 3, respectively, and showed no evidence of infectious virus at any timepoint. *Culex* MP-12, n= 22, 9 and 56 for days 1, 2, 3 respectively.

3.5 Viral growth curves in mosquito cell lines

To further characterize DDVax replication kinetics compared to MP-12 and ZH501 strains, growth curves were performed in three insect cell lines. Aag2 (*Ae. aegypti,* embryonic), ATC10 (*Ae. aegypti,* larval) and Ct (*Cx. tarsalis,* embryonic) cells were infected with DDVax, MP-12 or ZH501 over 6-day time courses. The *Aedes aegypti* larval cell line ATC10 was not susceptible to infection with any virus strain. DDVax replicated in Aag2 cells to lower peak titers than did MP-12 or ZH501 strains (Figure S5) (random effects mixed model ANOVA, p = 8.0e-4). Similarly, DDVax also attained lower titers than control viruses in Ct cells (random effects mixed model ANOVA, $p = 3.5e\text{-}4$). MP-12 grew to similar peak titers in Ct and Aag2 cells, at 9.1 and 9.5 $\log_{10}$ PFU/ml, respectively. Peak ZH501 titers were 8.0 and 6.9 $\log_{10}$ PFU/ml, in Ct and Aag2 cells, respectively. The virulent strain caused syncytial formation and lifting of cell monolayers, consistent with pathogenicity(Turell, Gargan, & Bailey, 1984), which could have affected final titers. Lastly, mean peak DDVax titers were 7.1 and 6.3 $\log_{10}$ PFU/ml, in Ct and Aag2 cells, respectively, which are lower than peak titers for MP-12. DDVax grew better in Ct cells than in Aag2 cells (Two way ANOVA, $p = 4.5e\text{-}5$), consistent with the mosquito data. While the calculated DDVax MOI was 0.01 for all cell lines, the actual MOI was 0.0052 for Aag2, 0.0127 for Ct, and 0.0153 for ATC-10 cells. The difference observed in replication kinetics of DDVax in the Ct cells as compared with the Aag2 cells may be due in part to the actual MOI being half the calculated value for Aag2 cells (Figure S5).

4 Discussion

This study utilized multiple approaches to demonstrate the relative safety of the DDVax vaccine candidate from the perspective of relevant mosquito species transmissibility and regulations regarding potential environmental impacts following field-use. These experiments were designed as part of a series of safety studies required prior to human clinical trials. DDVax showed favorable environmental safety profiles (e.g., low mosquito dissemination, and impaired transmission from inoculated livestock) compared to MP-12 vaccine and the wild-type parental virus, ZH501. Mosquitoes in two epidemiologically-relevant genera were challenged with viral titers up to 2 to 5 $\log_{10}$ PFU/ml higher than mosquitoes would be expected to encounter

in the field from vaccinated animals, and there was only one questionably positive transmission event. In a previous study, sheep vaccinated with DDVax did not develop any detectable vaccine-associated viremia following inoculation, suggesting that the overall burden of DDVax in animals is very low (Bird et al., 2011). Additionally, DDVax viral RNA copy numbers in bodies and legs/wings were significantly reduced in both *Aedes* and *Culex* compared to those infected with either MP-12 or ZH501 (Figure 2). This result is consistent with previously observed impaired viral dissemination phenotype in mosquitoes due to the deletion of the NSm coding region (Crabtree et al., 2012; Kading et al., 2014). Only one of 140 mosquito saliva samples contained live DDVax virus (Table 2), which was also consistent with previous experiments(Crabtree et al., 2012). This single positive saliva sample showed a single plaque, which may not have been infectious and for which we cannot rule out the possibility that it represents low-level contamination. Expected virus infection rates in these mosquito species have previously ranged between 63-84% for virulent recombinant ZH501 (rZH501) for *Ae. aegypti* (Crabtree et al., 2012; Kading et al., 2014), 58 – 95% for *Cx. tarsalis* (Bergren, Borland, Hartman, & Kading, 2021; Turell, Wilson, & Bennett, 2010) with midgut titers of over 6 $\log_{10}$ PFU in actively infected *Cx. tarsalis* (Bergren et al., 2021). Similarly, up to 100% infection occurred MP-12 infected *Cx. pipiens* (Turell & Rossi, 1991). In contrast, we expected 0% infection with DDVax infected*Ae. aegypti* (Crabtree et al., 2012). Overall, we observed similar results between ZH501 and MP-12 strains, with a significant reduction in infection of mosquitoes with DDVax.

While DDVax RNA was detectable in multiple body compartments of the mosquito, infectivity was very reduced or nil given the low RNA copy number detected in mosquitoes 14 days post *in vitro* infection (Figure 1B). For example, if mosquitoes imbibed a 5 µl blood meal of 8 $\log_{10}$ PFU/ml, then 5.7 $\log_{10}$ PFU would have been acquired. In our study, after two weeks incubation, 2.9 $\log_{10}$ mean RNA copies were detected in *Culex*bodies, 1.8 $\log_{10}$ RNA copies in legs/wings and 1.5 $\log_{10}$ RNA copies saliva (Figure 1B), suggesting that the virus may have somehow disseminated and persisted at a low level, but was not actively replicating in the mosquitoes. By comparison, mosquitoes of each species exposed to MP-12 and ZH501 had RVFV RNA copy numbers between 7-8 $\log_{10}$ by 14 days post-exposure (Figure 1B) after exposure to a blood meal containing greater than an order of magnitude less virus than that of DDVax (Figure 1A). This pattern was consistent with the results of the dose response experiment, in which the RNA copy number in different tissue compartments seems to be relatively stable after 14 days across all three exposure doses (Figure S3). It is not clear at this time how this spread would be occurring. Further, it is expected that RNA copy numbers would exceed infectious titers rendering the truly infectious virus population even lower(Wichgers Schreur et al., 2021).

Consistent with these findings, Kading et al.(Kading et al., 2014) reported 80% infection and 60% dissemination rates of rZH501 by*Ae. aegypti* mosquitoes, compared with 0% infection and 0% dissemination rates of the rZH501-delNSm (NSm deletion) strain, by plaque assay. Nevertheless, in rZH501-delNSm infections, viral protein was detected in most mosquitoes by immunofluorescence assay (IFA), consistent with viral protein translation with defective packaging or dissemination. Moreover, IFA foci in the midguts of mosquitoes infected with rZH501-delNSm were also very small compared with extensive midgut foci characteristic of rZH501(Kading et al., 2014). Therefore, detection of viral RNA (this study) and antigen (Kading et al., 2014) outside the midgut, in the absence of infectious virus, warrants further study.

Viral RNA detected in *Culex* saliva could be the result of cell-to-cell spread of DDVax through tissues in the absence of efficient viral assembly, or possibly "leakage" of virions from the alimentary tract in the absence of viral replication. Romoser and colleagues reported the particular affinity of virulent ZH501 RVFV for the cardia, intussuscepted foregut, fat body and salivary glands in *Culex pipiens* mosquitoes(Lerdthusnee, Romoser, Faran, & Dohm, 1995; Romoser, Faran, Bailey, & Lerdthusnee, 1992). The cardia and intussuscepted foregut are transitional tissues between the esophagus and the anterior midgut in the mosquito digestive tract(Romoser et al., 1992). Salivary glands are proximal to this region, embedded in fat body. One possible explanation is that DDVax retained similar tissue affinity in the absence of NSs and NSm, and, when combined with presumed less efficient viral assembly, led to detection of viral RNA but no infectious virus (Table 2, Figure 1B, S1 Table). In addition, Romoser et al. reported that, in *Culex* , RVFV ZH501 was able to escape to peripheral tissues as early as 1 day following an infectious blood meal (Romoser et

al., 1992), making it particularly rapid in disseminating compared to other arboviruses, eg., flaviviruses, which often require at least a week to reach the salivary glands(Sanchez-Vargas et al., 2009), depending on extrinsic incubation temperature. RVFV affinity for salivary glands was substantiated by the DDVax dose response experiment, in which nearly 19% of mosquitoes showed viral RNA in salivary expectorants at the lowest bloodmeal titer of 3.5 $\log_{10}$PFU/ml (Table S2). This level approached that of the presence of viral RNA in legs/wings.

To address concern about the one possible transmission event, *Cx. tarsalis* mosquitoes were subsequently challenged with artificial blood meals containing a range of viral titers. As expected, the percentage of mosquitoes that became infected, as determined by RNA genome copy number, decreased proportionally with the titer of DDVax in the artificial blood meal, but did not reach zero. The stable persistence of DDVax RNA in different tissue compartments was evident in all dosing groups (Figure S3). As experimentally predicted, the higher the blood meal titer, the higher the percentage of mosquitoes had detectable RNA, although infectious virus was not assayed in mosquitoes challenged with lower titer blood meals.

These results were further confirmed and placed into a realistic epidemiological context by feeding mosquitoes on inoculated goats. Infection of goats with wild-type ZH501 was not possible in this study due to biosafety considerations. Mosquitoes were fed on goats on days 1-3 post-inoculation with DDVax or MP-12. As expected, goats did not develop any detectable viremia, as determined by plaque assay. However, small ruminants, e.g. sheep, would be expected to develop a viremia ranging from ˜5-6 $\log_{10}$$TCID_{50}$/ml titers between 1-3 days post infection with a wild-type strain(Wichgers Schreur et al., 2021). Similarly, neither Wilson et al.(Wilson et al., 2014) nor Nyundo et al.(Nyundo et al., 2019) observed any detectable viremia in ruminants following vaccination with MP-12 strain. Morrill et al.(Morrill et al., 1991) noted a transient, low-titer viremia in lambs vaccinated with MP-12 strain. Sheep inoculated with DDVax failed to develop any detectable viremia (Bird et al., 2011). Therefore, it was surprising to observe that mosquitoes fed on these inoculated goats and held for seven days post-feeding developed infections (Figure 2, S3 Table).

Analysis on goat serum samples showed very low (<10 RNA copies /ml) RNA levels of RVFV in goat serum (Figure S4), which we interpreted to represent residual, circulating virus as opposed to actively replicating virus. The sensitivity of mosquito feeding was able to pick up this residual viral inoculum, however none of these mosquitoes developed a disseminated infection by 7 days post-exposure. For infection with ZH501 strain, dissemination has previously been documented to occur as early as 3 days post exposure(Romoser et al., 1992), with all mosquitoes having developed a disseminated infection by 10 days post-exposure(Kading et al., 2014).

Mosquito infectivity also becomes a function of volumetric constraints of mosquito blood meal size. While the probability of one mosquito imbibing infectious virions is lower at low virus titers, many mosquitoes imbibing a blood meal simultaneously would draw a larger collective volume of blood that could result in one or more mosquitoes picking up infectious virions. For example, detection of virus in a single mosquito blood meal is limited to titers >3 $\log_{10}$ PFU/ml serum, (approximately one PFU in one microliter of serum in a blood meal) (Kading et al., 2014). For a 25% probability of detecting virus in a single 2 µl mosquito blood meal, the serum titer needs to be 2.72 $\log_{10}$ PFU/ml (95% CI 2.19–3.27), while for a 50% probability of detection, the titer needs to be 3.64 $\log_{10}$ PFU/ml (95% CI 3.20–4.08)(Kading et al., 2014). Corresponding titers for 75% and 90% probabilities of detection were 4.56 $\log_{10}$ PFU/ml (95% CI 4.02–5.10) and 5.48 $\log_{10}$ PFU/ml (95%CI 4.71–6.24), respectively(Kading et al., 2014).

Wichgers Schreur et al.(Wichgers Schreur et al., 2021) documented the extraordinary efficiency of RVFV transmission between lambs and*Ae. aegypti* mosquitoes when using an animal model as opposed to an artificial system. Approximately 30% more RVFV saliva-positive mosquitoes resulted from feeding on viremic lambs than from feeding on a membrane system(Wichgers Schreur et al., 2021) testifying to the value of conducting these experiments with an *in vivo* model system to more realistically represent vertebrate infectiousness to mosquitoes. While dissemination of DDVax after our 7-day time point cannot be ruled out, our collective results suggest that transmission risk would be very low because any disseminated virions would not be infectious. In addition, based on previous reports, we expected a low combined probability of a

single mosquito imbibing an infectious virion precisely after inoculated and an extremely low imbibed virus titer. Moreover, impaired dissemination was due to the deletion of the NSm gene. Finally, we saw the lack of infectious DDVax expectorated in mosquito saliva even after a high titer virus challenge. These combined features combine to give DDVax a safe environmental profile.

4.2 **Conclusion**

Due to the double gene deletion of NSs and NSm, DDVax has less efficient viral replication in mosquitoes than a previous vaccine strain, MP-12 or wild-type ZH501. Mosquitoes were able to imbibe and harbor infectious DDVax following a high titer challenge in the lab or feeding on inoculated goats. However, DDVax replication and dissemination was impaired in mosquitoes, and only one individual mosquito had one DDVax plaque in its saliva after a high titer challenge. Given the combined probability of a single mosquito imbibing an infectious virion precisely after inoculation, the extremely low imbibed virus titer, the impaired dissemination in mosquitoes due to the deletion of the NSm gene, and the lack of infectious DDVax expectorated in mosquito saliva even after a high titer virus challenge, the transmission and dissemination of DDVax by mosquitoes from vaccinated individuals in an epidemiologically relevant scenario is highly unlikely.

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Ethics Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The US National Institutes of Health guidelines for the Care and Use of Laboratory Animals were followed.

Conflict of Interest

The authors declare they have no conflict of interest.

Supporting Information

Figure S1. Vaccinated goats were exposed to mosquitoes.

Figure S2. Depth of coverage across DDVax segments. Coverage depth was tabulated using samtools and depth in 10 nt windows is plotted.

Figure S3. Detection of DDVax viral RNA following exposure to limiting dilutions of virus at 14 dpi.

Figure S4. Viral RNA detected in goat blood at 1, 2, 3 days post-vaccination.

Figure S5. Mosquito cell culture shows reduced efficiency of viral replication for DDVax.

Table S1. RNA positive RVFV-exposed samples from *in vitro*mosquito challenge.

Table S2. RNA positive RVFV DDVax-exposed samples from virus serial dilution experiment.

Table S3. Proportion of mosquitoes with viral CPE in bodies at 7 days post-goat-exposure.

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Table 1: Single Nucleotide Variants.

Segment	Position (nt)	Coding impact	Reference Base	Variant Base	P1	P2	P3	P4	P5
L	4665	E1549E	A	G	0.00	0.07	0.09	0.12	0.16
L	5483	K1822M	A	T	0.04	0.03	0.03	0.03	0.04

Segment	Position (nt)	Coding impact	Reference Base	Variant Base	P1	P2	P3	P4	P5
L	5488	D1824Y	G	T	0.07	0.07	0.07	0.07	0.07
L	5513	R1832I	G	T	0.00	0.00	0.00	0.00	0.03
L	6113	Y2032C	A	G	0.05	0.05	0.05	0.04	0.03
M	31[a]	G3E	G	A	0.00	0.00	0.00	0.25	0.54
M	32 [a]	G3G	G	A	0.00	0.00	0.07	0.20	0.19
M	190	G56E	G	A	0.00	0.00	0.00	0.00	0.09
M	300	K93E	A	G	0.00	0.00	0.00	0.03	0.05
M	457	Y145C	A	G	0.00	0.00	0.00	0.06	0.00
M	462	L147I	C	A	0.00	0.06	0.09	0.09	0.00
M	499	G159D	G	A	0.00	0.00	0.00	0.11	0.55
M	808	R262K	G	A	0.00	0.07	0.07	0.09	0.00
M	818	K265N	A	T	0.00	0.00	0.00	0.00	0.04
M	925 [a]	N301I	A	T	0.00	0.00	0.03	0.03	0.00
M	926 [a]	N301K	T	A	0.06	0.34	0.40	0.49	0.90
M	1240	D406G	A	G	0.04	0.00	0.04	0.06	0.00
M	1473	P484S	C	T	0.00	0.00	0.00	0.07	0.00
M	2480	R819R	G	A	0.00	0.00	0.00	0.00	0.05

The variants at positions 31 and 32, and those at positions 925 and 926 are not linked.

Table 2 Proportion of mosquitoes with infectious virus in saliva following exposure to RVFV with an artificial membrane feeder.

Species	Virus	# samples	Saliva CPE positive (%)
Ae. aegypti	DDVax	120	0 (0%)
	MP-12	120	19 (16%)
	ZH501	118	32 (27%)
Cx. tarsalis	DDVax	120	1 (< 1%)
	DDVax	20*	0(0%)
	MP-12	120	115 (96%)
	ZH501	110	90 (82%)
	ZH501	15*	10 (67%)

*, no freeze-thaw