Title: Development of a Novel Non-human Primate Model for Rift Valley Fever

Running Title: Novel NHP model for RVF

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Rift Valley fever virus (RVFV) can cause severe human disease characterized by either acute-onset hepatitis, or delayed-onset encephalitis, or retinitis and blindness, or a hemorrhagic syndrome. The existing non-human primate (NHP) model for RVF utilizes an intravenous exposure route in rhesus macaques (Macaca mulatta). Severe disease in these animals is infrequent and large cohorts are needed to observe significant morbidity and mortality. To overcome these drawbacks, we evaluated the infectivity and pathogenicity of RVFV in the common marmoset (Callithrix jacchus) by intravenous, subcutaneous, and intranasal exposure routes to more closely mimic natural exposure. Marmosets were more susceptible to RVFV than rhesus macaques and experienced higher morbidity, mortality, viremia, and marked aberrations in hematological and chemistry values. Overwhelming infection of hepatocytes was a major consequence of infection by IV and SC exposure routes in marmosets. Additionally, these animals displayed signs of hemorrhagic manifestations and neurological impairment. Based on our results, the common marmoset model more closely resembles severe human RVF disease and is therefore an ideal model for the evaluation of potential vaccines and therapeutics.
Introduction

Rift Valley fever virus (RVFV) is a negative-sense, single-stranded RNA virus in the genus *Phlebovirus* (family *Bunyaviridae*). The virus was first isolated in 1930 in East Africa (15) and has since caused severe epidemics and epizootics throughout Africa and the Arabian peninsula (12, 31). Severe outbreaks have involved tens of thousands of both human and livestock cases for which no effective, commercially available human vaccines or antiviral drugs are available. Due to concerns regarding its use as a potential biological weapon, RVFV has been identified as a category A, high-priority select agent, by the National Institute for Allergy and Infectious Diseases (NIAID), the Centers for Disease Control and Prevention (CDC), and the United States Department of Agriculture (USDA).

RVFV is an arthropod-borne virus (arbovirus) that causes epizootics associated with abortion and high mortality in livestock during which humans become infected (31). Human infections result from infected mosquitoes (*Culex* and *Anopheles* mosquitoes appear to be the principal vectors for humans) or by contact with tissues, blood, or fluids from infected animals. After an incubation period of 2 to 6 days, an abrupt onset of fever, chills, and general malaise ensues. In most cases, human disease is mild, and recovery occurs without major consequences. Severe cases, which affect around 1-2% of infected individuals, are characterized by acute-onset liver disease, delayed onset encephalitis, retinitis, blindness, or a hemorrhagic syndrome, with a case fatality ratio of 10-20% in hospitalized individuals (26, 28, 30). Human cases have been reported in much of Africa, Saudi Arabia, and Yemen, with recent outbreaks in Kenya during 2006-2007 (12) and South Africa in 2008-2011(1).
The development of an effective vaccine or therapeutic to treat RVF in humans remains an important area of research. The United States Food and Drug Administration’s animal rule allows for the demonstration of drug or vaccine efficacy using animal studies instead of human clinical trials (44). This rule recommends the testing of potential vaccines and therapeutics in well described animal models. Ideally, this would involve an animal model using a non-rodent species such as a non-human primate (NHP). Several animal models of RVFV infection have been described. Mice are highly susceptible to infection with RVFV by subcutaneous (SC) or intraperitoneal (IP) injection leading to fulminant hepatitis and late-developing encephalitis (20, 22, 33, 37, 42). Similar to mice, hamsters and rats are also susceptible to infection (19, 20, 22, 37). Both hepatitis and encephalitis have been described in rats; however, their susceptibility to RVFV can vary significantly depending on the strain of rat used, and usually only one pathologic feature (i.e., hepatitis or encephalitis) is observed in a particular strain (5, 22, 37, 40). The hamster model has relied mainly on experimental infection with the related bunyavirus Punta Toro (3, 21), where only hepatitis (but not encephalitis) is the dominant pathologic feature. In addition, gerbils infected with RVFV reportedly develop uniformly fatal encephalitis in the absence of significant extraneural lesions (4).

In contrast to the majority of rodent models, infection of NHPs with RVFV does not seem to produce a uniformly fatal infection. The first study describing the infection of NHPs with RVFV was in 1931 (20), which reported that infection of rhesus macaques induced febrile responses and leukopenia but did not result in a fatal infection. A later study addressed the effect of different exposure routes such as IP, intracerebral (IC), SC, or intranasal (IN) inoculation of rhesus macaques using blood or tissues from infected animals

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as the inoculums. The NHPs became viremic, developed a fever, and leukocytosis was evident, followed by leukopenia, but no signs of disease resulted except for a decrease in activity during the peak of fever (19). Rhesus (17, 32) and cynomolgus (17) NHPs infected by the aerosol route appeared to be slightly more susceptible to infection compared to peripheral exposure routes (17). The susceptibility of other species of NHPs had been evaluated previously and four South American NHP species, two brown capuchin monkeys (Cebus fatuellus and Cebus chrysopus) and two common marmosets (Callithrix jacchus and Callithrix penicillata), were found to be more susceptible to infection than three African species, which included the green guenon (Cercopithecus callitrichus), the sooty mangabey (Cercocebus fuliginosus) and the Patas guenon (Erythrocebus patas) (18). Spider monkeys (Ateles ater) appeared to be refractory, (17) while baboons (Papio anubis) appeared to be comparable to rhesus macaques (16). However, it is important to note that these earlier studies utilized small numbers of NHPs. Therefore, further evaluation of these species with larger cohorts of animals was warranted.

To date, rhesus macaques infected with RVFV strain ZH501 appear to provide the most realistic model of human infection (37). In previous studies, rhesus macaques were usually infected IV with 5 log_{10} plaque forming units (PFU) of RVFV. The outcome of these infections can be broadly divided into three groups based on the observations of large cohorts of animals (usually 15-20 rhesus macaques): 1) fatally infected rhesus macaques that developed severe clinical disease and succumbed (~18%); 2) clinically ill rhesus macaques that survived (~41%); and 3) rhesus macaques that developed very mild or no apparent clinical illness and survived (~41%). Rhesus macaques with severe clinical disease developed signs of illness 2-4 days after infection characterized by anorexia, depression,
vomiting, and weakness. Coagulopathy was manifested by petechial and purpuric skin lesions on the face, ears, abdomen, and medial thigh. Animals that developed moderate clinical illness and survived were characterized by reduced appetite, cutaneous petechial hemorrhages on the abdomen and medial aspect of the thigh, and occasional vomiting (34).

Pathologic changes in the liver were shown to occur in the characteristic mid-zonal pattern, which is observed in humans (46) and other animals infected with RVFV (17-19, 35). Severe disease in rhesus macaques was accompanied by extensive liver necrosis, evidence of disseminated intravascular coagulation (DIC), and microangiopathic hemolytic anemia (14, 36, 38).

Human RVF presents as a spectrum of disease manifestations ranging from mild febrile episodes to death. The rhesus model faithfully represents this because the clinical disease syndromes are similar to those observed in human cases of RVF. However, using a moderately susceptible infection model is not ideal to demonstrate the efficacy of newly developed vaccines and therapeutics. Less than 20% of rhesus macaques infected with RVFV develop severe disease, and no ocular disease has been reported in this model. Additionally, IV infection does not represent a natural exposure route since RVFV infected mosquitoes primarily transmit virus extravascularly (45). Therefore, the development of a more susceptible NHP model using a more natural exposure route would be advantageous. In this study, we evaluated the susceptibility of the common marmoset (Callithrix jacchus) to RVFV. This New World primate species was chosen because it has been successfully used to study a number of other viral diseases caused by arenaviruses, herpesviruses, the coronavirus causing severe acute respiratory syndrome, and an alphavirus (2, 6, 13, 23, 24, 29). Here we describe the establishment of a new NHP model for RVF using the common marmoset,
which overcomes some of the major limitations of existing primate models. Marmosets were more susceptible to RVFV than rhesus macaques and experienced higher morbidity, mortality, viremia, and marked aberrations in hematological and chemistry values. These animals exhibited acute-onset hepatitis, delayed-onset encephalitis, and hemorrhagic disease, which are dominant features of severe human RVF.

Materials and Methods

Viral Strain, Animals, and Study Design

Recombinant viral strain ZH501 was rescued as previously described (10) and the exact complete genome sequence confirmed by techniques described by Bird et al. (11). Strain ZH501 was originally isolated from a fatal human case during the 1977 epidemic in Egypt.

Four healthy, adult, rhesus macaques (Macaca mulatta), 3 to 4 years old and ranging in weight from 4.8 to 5.7 kg were obtained from World Wide Primates and Shared Enterprises. Twenty healthy adult marmosets (Callithrix jacchus), 2 to 11 years old and ranging in weight from 279 to 547g were obtained from either the Institute of Chemical Defense (ICD), in Aberdeen Proving Ground, MD (original vendor was World Wide Primates) or Glaxo Smith Klein (GSK; original vendor was Harlan UK Hillcrest). None of these primates was exposed to any infectious pathogens in previous studies and all primates were determined to be RVFV naïve by plaque reduction neutralization test (PRNT; methods below) before the initiation of the study.

For the study design, four animals per exposure route received 7 log10 PFU/mL RVFV in 1 mL volume IV, SC (one cohort of four animals received 5 log10 PFU/mL), or IN. Two animals were included as uninfected negative controls: one animal to serve as a negative control for
injectable routes of exposure (IV and SC) and one animal to serve as a negative control for the IN exposure route. After RVFV exposure, all animals were monitored for temperature changes, weight loss, and survival, and blood samples were collected on days -3, 0-7 and once a week thereafter (until day 28-36 PI) for virological, hematological, immunological, and chemical analyses. Additionally, blood was collected from IV-exposed animals (day 0) within 5 minutes of virus inoculation to ensure IV exposure occurred. When the animals succumbed to infection or were euthanized, either when moribund or at the study endpoint (ranged from days 28-36 PI), tissues were collected for viral titer determination and histopathology.

Research was performed under an Institutional Animal Care and Use Committee-approved protocol in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in The Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where the research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

**Hematology, Blood Chemistries, and Virological Assays**

Whole blood was added to an EDTA tube (Sarstedt, Numbrecht, Germany) for complete blood count (CBC) determination using a Hemavet instrument (Drew Scientific, Dallas, TX) according to manufacturer’s instructions. Clinical chemistry analyses were performed by addition of whole blood to a lithium heparin tube (Sarstedt) using the comprehensive diagnostic panel analyzed on a Vetscan instrument (Abaxis, Union City, CA) according to manufacturer’s instructions. Normal ranges in the chemistry and hematology results of healthy rhesus macaques (27) and marmosets (47) were used as reference values. The plasma was then collected for viral
titer determination by standard plaque assay as previously described (42). At the time of necropsy, the following tissues were collected for viral titer determination: liver, cerebrum, spleen, kidney, lung, heart, adrenal gland, inguinal lymph node, axillary lymph node, mesenteric lymph node, duodenum, jejunum, ileum, ovaries/testis, skeletal muscle, bone marrow, and retina. Tissues were collected, weighed, and homogenized in EMEM containing 5% fetal bovine serum and gentamicin. Tissues were homogenized using the Qiagen Mixer Mill 300 (Retsch, Newtown, PA) then centrifuged at 9,000 x g for 10 min and the supernatant stored at -70°C until further evaluation. Tissues collected from moribund animals were titrated by standard plaque assay. Tissues collected at the study endpoint were homogenized according to the methods above and a 1:10 dilution of the supernatant added to 24-well plate of Vero cells in duplicate in a volume of 100 μL for each well. Plates were incubated for 1 h at 37°C with rocking every 15 min. After the incubation, 0.5 mL of EMEM was added to each well and incubated for 4 days to monitor for cytopathic effects (CPE).

**Histopathology**

Full necropsies and histological examination were performed by a board-certified veterinary pathologist. The following tissues were collected during necropsy: Axillary, inguinal, submandibular, mesenteric and tracheobronchial lymph node; submandibular salivary gland; haired skin; brachial plexus; sciatic nerve; skeletal muscle; bone marrow (femur); eyes; brain; pituitary gland; spleen; adrenal gland; kidney; liver; stomach; duodenum; pancreas; jejunum; ileum; cecum; colon; testis/ovary; prostate gland/uterus; urinary bladder; tongue; tonsil; trachea; esophagus; thyroid gland; lung; thymus; and heart. All collected tissues were immersion-fixed in 10% neutral buffered formalin for at least 21 days. The tissues were trimmed and processed according to standard protocol (39). Histology sections were cut at 5 to 6 μm on a rotary...
microtome, mounted on glass slides, and stained with hematoxylin and eosin. For immunohistochemical analysis, serial sections of tissue were cut and stained for RVF antigen using a mouse monoclonal antibody (4D4) against the glycoprotein Gn (7, 25) and an immunoperoxidase assay system (EnVision; DAKO). Normal hepatic tissue served as the negative control; the positive control tissue was liver from a known RVF-positive animal. Normal mouse IgG was used as the negative serum control for the control slides. For the immunohistochemistry study, the unstained tissue sections were deparaffinized, rehydrated, subjected to methanol-hydrogen peroxide block, rinsed, and pretreated with Tris/EDTA buffer at 97°C for 30 min. A serum-free protein block (DAKO) plus 5% normal goat serum was applied for 30 min. The primary antibody was then applied to the tissue at a dilution of 1:100 and incubated at room temperature overnight. The tissue sections were rinsed and then exposed to the EnVision horseradish peroxidase labeled polymer for 30 min at room temperature. All sections were exposed to DAB permanent chromogen for about 5 min, rinsed, counter-stained with hematoxylin, dehydrated, and applied a coverslip with Permount.

Serology

Anti-RVFV total IgG ELISA was performed essentially as described previously (9), with the following modifications necessary for NHP specimens. BHK cell lysate was used rather than Vero E6 cells and the secondary goat anti-monkey IgG horseradish peroxidase-conjugated antibody (KPL, 074-11-021), which was raised in rhesus macaques and most likely contributed to low adjusted sum OD values. Neutralizing antibodies were assayed in serum for rhesus macaques and plasma for marmosets with a 50% PRNT as previously described (8).

Statistical Analysis
Repeated measures ANOVA was used to compare chemistry, viremia, percent change in weight and percent change in temperature over time and between groups. Due to decreasing sample sizes, analysis was limited to days 0 through 7. All analyses were conducted using SAS version 9.2.
Results

Marmosets were infected with RVFV by three exposure routes, IV (7 log_{10} PFU), SC (two doses, 5 and 7 log_{10} PFU), and IN (7 log_{10} PFU) and rhesus macaques were exposed IV to 7 log_{10} PFU of RVFV for a direct comparison with the current NHP model. The percent survival (Figure 1), viremia (Figure 2), weights (Figure 3), body temperatures (Figure 4), blood chemistry, and CBC values (Figures 5-6, Supplemental Figures 1-3) were determined for all animals throughout the study.

Survival. None of the rhesus macaques (n=4) succumbed or presented with clinical illness (Figure 1). In contrast, marmosets exposed IV (n=4) had one animal that succumbed on day 2 postinfection (PI) whereas two others presented with clinical illness, which was characterized by anorexia, decreased activity, and ruffled fur/hunched posture. Half of the marmosets that were exposed SC (n=4) succumbed or were euthanized and three out of four of the animals presented with a clinical illness similar to that described for marmosets exposed IV. For marmosets that received 7 log_{10} PFU of RVFV, one animal succumbed on day 4 PI and another on day 11 PI. For marmosets that received 5 log_{10} PFU of RVFV, one animal was euthanized on day 7 and another on day 12 PI. The marmoset that was euthanized on day 12 PI had signs of neurological impairment. This animal appeared disoriented, was shaking and falling over in its cage. When marmosets were exposed to RVFV IN, 100% mortality resulted and the marmosets succumbed or were euthanized on day 8, 9, and 11 PI and all of these animals presented with signs of neurological impairment as described above.

Viremia and Clinical Observations
Rhesus macaques and marmosets exposed IV. All rhesus macaques did develop a viremia, which peaked on day 2 PI (Figure 2A). All of the marmosets exposed IV developed viremia except for the animal that succumbed on day 2 PI (Figure 2B) and the presentation of clinical illness correlated with viremia. Viremia in marmosets exposed IV peaked on day 1 PI and when compared to the peak day of viremia in rhesus (day 2 PI), marmosets had on average 1 log_{10} PFU higher levels of viremia (p=0.27). Over the course of the study, there were no significant changes in the weights of the rhesus macaques (Figure 3A) and the marmosets exposed IV (Figure 3B; p=0.35). The temperature of rhesus macaques (Figure 4A) and marmosets exposed IV (Figure 4B) did not differ significantly throughout the study (p=0.57). One rhesus macaque had a slight increase in temperature, which peaked on day 6 PI and one marmoset had a slight decrease in temperature on day 4 PI, but the general trend remained similar. The liver enzyme alanine aminotransferase (ALT) peaked on days 2-3 PI in both rhesus and marmosets exposed IV (Figure 5A-B). However, the increase was more significant in marmosets overall compared to rhesus macaques (p=0.03). The WBC levels varied in rhesus and marmosets exposed IV (Supplemental Figure 1A-B). The blood urea nitrogen (BUN) and creatinine (CRE) values in rhesus macaques and marmosets exposed IV did not change significantly (Supplemental Figure 2A-B and Supplemental Figure 3A-B; p=0.39 and p=0.70 respectively).

Marmosets exposed SC. All animals developed viremia (Figure 2C) with the marmosets that were exposed with 5 log_{10} PFU of RVFV SC having the highest levels (p=0.04). Viremia persisted until the animals succumbed or were euthanized and the level of viremia correlated with presentation of signs of clinical illness as described above for animals exposed IV. An overall decrease in the weights was observed for marmosets exposed SC (Figure 3C;
p=0.04). In contrast to marmosets exposed IV, marmosets that were exposed SC had decreases in 
temperature in animals that succumbed or were euthanized (Figure 4C). The ALT (Figure 5C) 
and ALP (Figure 6C) levels were the highest in the animals exposed SC compared to the other 
exposure routes (p=0.02 for both). The WBC levels varied in marmosets exposed SC 
(Supplemental Figure 1C) showing a sporadic increase and decrease for most animals. In 
contrast to the IV exposure route, the BUN and CRE levels increased in marmosets exposed SC 
(Supplemental Figure 2C and Figure 3C; p<0.01 and p=0.79, respectively) suggesting kidney 
disease in these animals. Among the marmosets with notable increases, the BUN:CRE ratios 
ranged from 24-78, which provides further evidence for the occurrence of kidney disease in these 
animals. However, one marmoset had BUN:CRE ratios that ranged from 50-148, which suggests 
dehydration occurred in this animal.

**Marmosets exposed IN.** All marmosets exposed IN developed viremia (Figure 
2D), which peaked on day 2 or 3 PI and declined steadily until the animals succumbed or were 
euthanized. In contrast to marmosets exposed IV and SC, marmosets that were exposed IN did 
show a significant decrease overall in weight after exposure (Figure 3D; p<0.01). Similar to 
marmosets that were exposed SC, the marmosets exposed IN had decreases in temperature in 
animals that succumbed or were euthanized (Figure 4D; p=0.66). The ALT (Figure 5D) and ALP 
(Figure 6D) levels were elevated in the animals exposed IN, but to a lesser extent than IV and SC 
exposed animals. In contrast to the other exposure routes, marmosets exposed IN had a 
significant increase in WBC on day 1 PI for three out of four animals (Supplemental Figure 1D; 
p=0.02). Similar to the marmosets exposed SC, the BUN and CRE levels increased in marmosets 
exposed IN (Supplemental Figure 2D and Supplemental Figure 3D; p<0.01 and p=0.67,
respectively) suggesting kidney disease in these animals. However, the BUN:CRE ratios ranged from 55-245, which suggests these animals were dehydrated.

**Viral Titers in Tissues.** Tissues were collected at the time of necropsy and the viral titers determined by standard plaque assay in the animals that succumbed or were euthanized (Supplementary Table 1). Virus was found in the liver of all marmosets exposed IV and SC, but only in two out of four of the animals exposed IN. Virus was found in the cerebrum of six out of nine of the marmosets exposed by all three exposure routes. The kidney was the only tissue where virus was detected in all marmosets. It is interesting that virus was detected in the retina of two of the marmosets exposed SC and all marmosets exposed IN. Other tissues where virus was detected in marmosets depending on the exposure route included the spleen, lung, heart, adrenal gland, inguinal, axillary and mesenteric lymph nodes, duodenum, jejunum, ileum, ovaries/testis, skeletal muscle, and bone marrow. Tissues were also collected at the time of necropsy for the surviving animals at the study endpoint (days 28-35 PI) and analyzed for virus by cytopathic effect assay where all tissues were found to be negative for virus (data not shown).

**Pathology findings.** The primary gross pathology finding identified in marmosets that succumbed to infection by the IV or SC routes of exposure was an enlarged (about 2-3 times the size of an uninfected control), yellow-orange, friable liver (Figure 7A-B). The histopathologic findings in the liver were characterized by hepatocellular degeneration and necrosis that primarily affected the centrilobular to midzonal areas (Figure 7C). Degenerate hepatocytes often contained variably sized, clear, round vacuoles (lipid) in the cytoplasm. Scattered among necrotic hepatocytes were brightly eosinophilic, round bodies interpreted as Councilman-like bodies (remnants of apoptotic hepatocytes). Rarely, eosinophilic intranuclear inclusion bodies were observed in hepatocytes. Immunohistochemically, hepatocytes exhibited intense positive
staining for RVFV antigen (Figure 7D). No directly attributable pathological changes in the liver were noted in the rhesus macaques exposed to RVFV.

An additional histologic finding observed in the marmoset exposed to RVFV by IV route that succumbed on day 2 PI was the presence of fibrin deposition and fibrin thrombi in several tissues (kidney, lung, choroid layer of the eye, and brain), suggesting that DIC was present at the time of death (Figure 8). None of the other animals that succumbed to infection by any other route of exposure exhibited evidence of DIC.

Gross lesions were not observed in the brain of any of the marmosets that succumbed to infection by the multiple exposure routes. However, histopathological changes were noted in the brains of marmosets exposed SC that succumbed on days 11 and 12 PI and all marmosets exposed IN. Representative histopathological changes portrayed widespread brain lesions with moderate numbers of inflammatory cells consisting primarily of lymphocytes and neutrophils affecting the gray matter (encephalitis), changes that were not found in uninfected controls (Figure 9A-B). Neuronal necrosis, satellitosis (surrounding of neurons by glial cells), gliosis (reactive glial cells), and neuronophagia (phagocytosis of degenerate/necrotic neurons) were also commonly observed. Immunohistochemically, there was intense multifocal positive staining of neurons for RVFV antigen (Figure 9C-D). For rhesus macaques, one out of four animals displayed minimal to mild multifocal lymphoplasmacytic inflammation of the brain accompanied by gliosis at the study endpoint. Interestingly, one of the SC-exposed marmosets that succumbed to RVFV on day 11 PI exhibited widespread positive staining for RVFV antigen in the retina of the eye without histologic changes (Figure 10). However, positive antigen staining of the retina was not observed in any of the other marmosets that succumbed to RVFV despite virus being detected by cytopathic assay (Table 1) as discussed above.
The histopathology analysis focused on the liver, brain, and retina because these have been shown to be important RVFV targets for other animals. However, other gross and/or histological changes were noted in the lymphoid tissues, adrenal gland, kidney, and heart. The lungs were also noted as a target tissue in marmosets exposed IN. A detailed study is currently underway to further characterize the pathology of RVFV in these tissues.

Serology. All rhesus macaques and some marmosets developed neutralizing antibody titers as early as day 7 PI (Supplementary Table 2). By day 14 PI, all animals sampled developed neutralizing antibody titers except for the uninfected controls. On day 14 PI, all rhesus macaques and some marmosets developed RVFV IgG titers, which increased steadily until the study endpoint (Figure 11). The SC exposed animals that were euthanized on days 11 and 12 PI developed neutralizing antibody titers, but only one animal developed a RVFV IgG titer (Figure 11C). For marmosets that succumbed to RVFV IN, all but one developed neutralizing antibody titers either before or on the day of euthanasia, (note, some samples were not collected because the animal succumbed to infection before sample collection), but none of the animals developed a RVFV IgG titer (Figure 11D).

Discussion

The results of this study identified the common marmoset as a useful model of severe human RVF for the evaluation of potential vaccines and therapeutics. The manifestations of hemorrhagic disease, acute-onset hepatitis, and delayed-onset encephalitis observed in these marmosets are similar to the most severe consequences of RVFV infection in humans. Accordingly, the marmoset model could be useful for investigating key processes in the
pathogenesis of severe RVF. We identified a number of advantages for using this new primate model, which overcomes limitations of the existing rhesus macaque RVF primate model.

Overall, marmosets were more susceptible to infection with RVFV and had more marked changes in their clinical chemistry and hematology values than rhesus macaques. Viremia was observed in all animals, and clinical illness did correlate with higher levels of viremia in marmosets. The mortality ratio for all the marmosets ranged from 25-100% with 50-100% showing signs of clinical illness depending on the route of exposure. This is in contrast to no mortality or signs of clinical illness in any of the rhesus macaques. Previous studies reported that a minority of rhesus macaques exposed to RVFV do succumb to infection and have signs of clinical illness (37), but these studies used large cohorts of animals (15-20) and we evaluated a smaller cohort of four animals. A major advantage of the marmoset RVF primate model is the manifestation of severe disease in a small cohort of animals. Marmosets therefore become a cost beneficial model, which is enhanced by their lower purchase price than that of rhesus macaques.

The earliest fatality observed was in the marmosets exposed to RVFV IV. One marmoset succumbed on day 2 PI and had histological evidence of DIC, suggested by thrombocytopenia and fibrin thrombi. These features are also observed in human hemorrhagic infections of RVFV (41) and have also been reported to occur in rhesus macaques exposed IV (14), but they were not observed in the rhesus macaques used in this study, most likely due to the small cohort of animals. Evidence of DIC was only observed in the marmosets exposed IV and not in the other routes of exposure. This suggests that marmosets exposed IV may serve as a beneficial model for human hemorrhagic RVF disease.
Marmosets exposed to RVFV SC developed both hepatitis and encephalitis and, in one instance, viral antigen was detected in the retina by immunohistochemistry. Marmosets euthanized on day 4 PI (7 log_{10} PFU exposure) and day 7 PI (5 log_{10} PFU exposure) appear to have succumbed from acute-onset hepatitis whereas marmosets euthanized on day 11 PI (7 log_{10} PFU exposure) and day 12 PI (5 log_{10} PFU exposure) succumbed from late developing encephalitis. The route of neuroinvasion in these marmosets is unclear and will require serial sampling studies to evaluate the spread of the virus over time. The meningoencephalitic form of RVF occurs in less than 1% of natural human cases (26), but residual CNS symptoms are common in neurological cases, and may be severe. In the context of the tens of thousands of human cases that may arise during outbreaks, even a low percentage of neurological cases can provide a significant medical challenge. Therefore, future studies in the marmoset model would be useful to evaluate the route of neuroinvasion in hopes of better understanding this severe form of human RVF disease.

The marmoset exposed to RVFV SC that succumbed on day 11 PI also exhibited widespread positive immunohistochemical staining in the retina of the eye although there was no associated inflammation. Even though virus was detected by plaque assay in the retina of several other marmosets, this was the only case detected by immunohistochemistry. This is likely due to the differing sensitivities of the assays and should be addressed further to determine if the marmoset model will be useful to investigate RVFV associated retinitis.

The highest levels of viremia were in the fatally infected marmosets exposed SC with 5 log_{10} PFU of RVFV. It is interesting that the animals that received the lower dose of virus (5 vs. 7 log_{10} PFU) developed the higher level of viremia. RVFV infected mosquitoes have been shown to primarily transmit around 3 log_{10} PFU of virus extravascularly (45), so our results suggest that...
a lower virus dose delivered peripherally could potentially cause a higher level of viremia. We speculate that this could be due to the virus actively replicating to higher levels at the inoculation site prior to an active immune response being initiated to clear the virus. The neutralizing antibody titers were slightly higher in the marmosets exposed to RVFV SC at the lower dose. Marmosets that were exposed to RVFV SC and succumbed or were euthanized seemed to develop a slightly delayed neutralizing antibody and RVFV IgG response than marmosets that survived infection, which may have influenced their survival.

Marmosets exposed SC had the lowest body temperatures at the time of euthanasia. Fevers were never noted in these animals, but could have been missed since temperature was not continually monitored and also because body temperature is known to decrease as major organ systems begin to shut down. In fact, temperature decrease was drastic shortly before death and neared room temperature in fatally infected marmosets exposed SC at the higher virus dose. These animals also had the most significant increases in the liver enzymes ALT and ALP, which signified that widespread hepatocellular damage occurred in these animals and contributed to lethality.

Interestingly, marmosets exposed IN had the highest level of morbidity and mortality compared to the other exposure routes. All of the animals infected IN succumbed from a late developing encephalitis and interstitial pneumonia, and IN-exposed marmosets where the only cohort where a consistent decrease in weight and increase in WBC was observed. Most of these animals also had a decrease in body temperature and an increase in liver enzymes similar to SC exposed marmosets. The observed leukocytosis was most likely due to the high degree of inflammation caused by the virus. Leukocytosis is known to occur during the early phase of recovery in RVFV- infected livestock (41).
Although RVFV is transmitted most often through the bite of an infected mosquito, it can also be transmitted by aerosol, which is evident by the number of laboratory workers who have become infected (43) and the potential for infection of veterinarians and abattoir workers who handle infected animals (although infection can also occur through mucosal contact in this setting). The amount of virus associated with infected animal tissues, body fluids, aborted fetal materials, and placental membranes is very high (>6 log<sub>10</sub> PFU/mL) and allows for the adequate generation of aerosols and contact contamination, which can account for many human infections (37). The incidence of CNS manifestations resulting from an aerosol infection has been speculated to be much greater than is typical with peripheral infection given the potential for neuroinvasion to occur more easily through aerosol exposure. Our results with the marmoset model suggest that they are more susceptible to neurological manifestations when exposed by aerosol compared to peripheral exposure routes. Marmosets exposed IN offer a novel model to further investigate the specific mechanism of neuroinvasion by RVFV and could be compared to the mechanism of neuroinvasion caused by a peripheral exposure (i.e., SC).

In conclusion, this newly described marmoset model represents a novel NHP model that mimics the severe manifestations of human RVF for the evaluation of potential therapeutics and vaccines. The major pathological features of hemorrhagic disease, hepatitis, encephalitis, and possibly retinitis are reproduced in marmosets depending on the route of exposure and can be exploited to further understand RVFV induced pathology. Future work should follow the progression of disease in this model and further evaluate the immune response to RVFV in marmosets.

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**Figure Legends**

**Figure 1.** Percent survival of rhesus macaques exposed IV and marmosets exposed IV, SC, and IN with RVFV (n=4).
Figure 2. Viremia in A) rhesus macaques exposed IV and marmosets exposed B) IV, C) SC, and D) IN with RVFV (n=4). The asterisk indicates an animal that succumbed or was euthanized.

Figure 3. Percent change in baseline of the weight of A) rhesus macaques exposed IV and marmosets exposed B) IV, C) SC, and D) IN with RVFV (n=4). The asterisk indicates an animal that succumbed or was euthanized.

Figure 4. Percent change in baseline of the temperature of A) rhesus macaques exposed IV and marmosets exposed B) IV, C) SC, and D) IN with RVFV (n=4). The asterisk indicates an animal that succumbed or was euthanized.

Figure 5. Percent change in baseline of the alanine aminotransferase (ALT) levels in the blood of A) rhesus macaques exposed IV and marmosets exposed B) IV, C) SC, and D) IN with RVFV (n=4). The asterisk indicates an animal that succumbed or was euthanized. The box represents normal ALT reference range variability in healthy animals.

Figure 6. Percent change in baseline of the alkaline phosphatase (ALP) levels in the blood of A) rhesus macaques exposed IV and marmosets exposed B) IV, C) SC, and D) IN with RVFV (n=4). The asterisk indicates an animal that succumbed or was euthanized. The box represents normal ALP reference range variability in healthy animals.

Figure 7. Gross, histologic, and immunohistochemical findings in the liver of RVFV infected marmosets. A). Gross image of the liver from an uninfected control marmoset. B). Gross image of the liver of a RVFV infected marmoset exposed IV on day 2 PI. C). Hematoxylin and eosin stain. Hepatocellular degeneration and necrosis in the liver of a SC-exposed marmoset on day 4.
Figure 8. Histologic findings consistent with DIC in the marmoset exposed to RVFV IV that succumbed on day 2 PI. A). Hematoxylin and eosin (HE) stain of the kidney showing a fibrin thrombus in vessel (delineated by asterisks). B). HE stain of the lung showing a fibrin thrombus in vessel (delineated by an asterisk). C). HE stain of the choroid layer of the eye showing fibrin thrombi in the vessels (delineated by arrows). D). HE stain of the pons region of the brain showing an acute infarct (delineated by arrows) with a fibrin thrombus in an adjacent blood vessel (delineated by an asterisk).

Figure 9. Histologic and immunohistochemical findings in the brain of RVFV infected marmosets. A). HE stain of the hippocampus region from an uninfected control marmoset. B). HE stain demonstrates increased cellularity with perivascular inflammation in the hippocampus of a 5 log10 PFU SC-exposed marmoset that was euthanized on day 12 PI. The inset is a magnified view of the same region showing necrotic neurons surrounded by numerous neutrophils. C). Immunohistochemistry demonstrates the amount of RVFV antigen in the hippocampus of the same SC-exposed marmoset that was euthanized on day 12 PI. D). A higher magnification of the immunohistochemistry of the hippocampus demonstrating intense immunoreactivity of neurons for RVFV antigen.

Figure 10. Immunohistochemical findings in the retina of a 7 log10 PFU SC-exposed marmoset that was euthanized on day 11 PI. Neurons and associated fibers are shown to be multifocally positive for RVFV antigen.
Figure 11. Results of anti-RVFV total IgG adjusted $\text{Sum}_{\text{OD}}$ ELISA of sera from A) rhesus macaques exposed IV and marmosets exposed B) IV, C) SC, and D) IN with RVFV (n=4).