CHARACTERIZATION OF THE HUMORAL IMMUNE RESPONSE IN RATS AND NON-HUMAN PRIMATES EXPOSED TO AEROSOLIZED VIRULENT RIFT VALLEY FEVER VIRUS

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ABSTRACT

Rift Valley fever virus (RVFV) is an emerging zoonotic pathogen that has been responsible for extensive and devastating outbreaks of disease in both humans and livestock throughout Africa and the Arabian Peninsula. Humans infected with RVFV can recover after a brief febrile illness or go on to develop a more severe outcome including encephalitis, hepatitis, or hemorrhagic fever. Although human transmission primarily occurs through direct interaction with sick animals, inhalational infection can occur, making aerosolized RVFV a plausible bioweapon with potential for severe public health consequences. RVFV does not have a well-characterized rat or non-human primate (NHP) model for aerosol challenge. Animal models are essential for the testing of medical countermeasures, with knowledge of the host immune response aiding in their development. To further our understanding of the role that antibodies play in shaping the outcome of respiratory disease, inbred rats and non-human primates were exposed to aerosolized RVFV. Wistar-Furth, ACI, and Lewis rats were challenged in median lethal dose and serial sacrifice studies from which samples were tested to determine the robustness and timing of the

IgG response. Wistar-Furth rats succumbed to hepatic disease shortly after infection, and never mounted a detectable antibody response. ACI and Lewis rats developed neurologic disease, with IgG appearing 6 d.p.i. and potentially influencing host survivability. To investigate the significance of the humoral response during respiratory infection of NHPs, cynomolgus macaques, rhesus macaques, African Green monkeys, and marmosets, were inoculated with RVFV via aerosol route, with blood samples taken at several time points. Cynomolgus and rhesus species were not sensitive to developing disease, but elicited strong IgG and neutralizing antibodies in response to inoculation. AGMs and marmosets showed moderate to high susceptibility to neurologic disease, even in the presence of extremely high titers of neutralizing antibodies. Further immunity studies are pertinent to better comprehend these host-pathogen interactions after RVFV aerosol challenge.

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1.0 INTRODUCTION

Rift valley fever virus (RVFV) is an enveloped, single-stranded RNA virus of the *Bunyaviridae* family and *Phlebovirus* genus[1]. Its segmented genome is approximately 11.9 kilobases in length[2], in which two segments (L and M) are negative sense, and the other segment (S) is ambisense[3]. The virus encodes 6 proteins: the viral RNA polymerase (L segment), the Gn and Gc glycoproteins (M segment), NSm (M segment), NSs (S segment, complementary orientation), and the nucleoprotein (S segment, antisense orientation)[4].

RVFV is zoonotic pathogen that infects both animals and humans and is transmitted primarily by mosquitoes[5]. Ruminants are most susceptible to infection, with epizootics characterized by abortion storms and neonatal mortality nearing 100%[6]. The adult livestock mortality rate is approximately 10-20%[7]. Humans can be infected with the virus through a bite from an infected mosquito or by percutaneous or aerosol routes following exposure to infected animal tissue[8]. Although most humans get a self-limited febrile illness, 1-2% of those infected will develop severe disease with the case fatality rate of the affected persons being approximately 10 to 20%[910].

Although outbreaks have occurred only in Africa and the Arabian Peninsula[11], the virus can utilize dozens of mosquito species that are present in North America and Europe[12]. The virus could be introduced to virgin soil accidentally by international travel or importation of infected animals and mosquitoes, or deliberately through acts of bioterrorism. The CDC, USDA, and WHO consider RVFV to be a major zoonotic threat due to its potential for severe

consequences and international spread[13]. The result of an outbreak on virgin soil could cause significant direct and indirect consequences for the affected areas.

1.1 RIFT VALLEY FEVER VIRUS

1.1.1 Epidemiology and transmission

Rift Valley fever virus was first reported by veterinarians and livestock officers in the early 1900s[14], but it wasn't until 1931 that the virus was isolated during an outbreak in lambs and ewes in the Great Rift Valley of Kenya[15]. Since then, the virus has been responsible for devastating epidemics in regions of Africa, including Kenya[16], Tanzania[17], Sudan[18], Egypt[19], and Mauritania[20], and more recently, Yemen[21] and Saudi Arabia[22].

RVFV is most commonly found in areas where livestock is raised, such as eastern and southern Africa, but the virus also exists in most sub-Saharan countries, as well as Madagascar[23]. The first outbreak that occurred outside of Africa was reported in 2000 in the Arabian Peninsula[24].

Multiple human disease outbreaks have occurred in the past several decades. An epidemic in 1977 in Egypt caused approximately 18,000 cases of disease and resulted in nearly 600 deaths[25]. An outbreak in Senegal-Mauritania in 1987 caused infection in 89,000 individuals, with more than 200 deaths reported[26]. Sudan experienced an outbreak in 2007, with a total of 747 laboratory confirmed cases and 230 recorded deaths. The total number of human cases during the Sudan outbreak is estimated to be close to 75,000[27], but due to the poor health

infrastructure in rural areas, many cases of RVFV infection go unreported, as many people never seek medical attention for an accurate diagnosis[28].

RVFV primarily infects livestock including sheep, cattle, buffalo, camels, and goats, but is also capable of infecting dogs, cats, horses, monkeys, rodents, and humans[29]. Virus amplification occurs primarily in sheep and cattle[30]. The virus is incredibly pathogenic in livestock, with the most notable livestock outbreak occurring in Kenya in 1950-1951, which resulted in the death of 100,000 sheep and 500,000 abortions[31]. Neonatal and fetal mortality can reach nearly 100%, as the virus is known to be transmitted *in utero*. In 2006, the first report of *in utero* transmission to a human fetus was described[32].

The main route of transmission for livestock is through a variety of mosquito vectors, specifically *Aedes*, *Culex*, and *Anopheles* species[33]. The virus has been isolated in over 40 mosquito species, in 8 genera[34]. Several of the susceptible vectors are present in Europe and North America, making international expansion of the virus a serious concern. Florida has been suggested as an optimal location for the virus to inhibit due to its tropical and sub-tropical regions and capable mosquito vectors[35].

Epizootics of Rift Valley fever typically occur during years of excessive rainfall and flooding. The virus can lay dormant in competent mosquito reservoirs during inter-epidemic periods, but large amounts of rainfall cause explosive hatching of infected mosquito eggs. The mosquitoes then feed on livestock, transferring and amplifying the virus, causing the disease to spread[36]. Humans are primarily infected through contact with sick livestock, but can also contract the disease through mosquito bites or through aerosol exposure to blood, body fluids, or tissues from infected animals[37]. Slaughterhouse workers and those handling sick animals are

most at risk for infection during outbreaks, and make up the majority of inhalational Rift Valley Fever cases[38]. Accidental laboratory aerosol exposures have been reported a number of times[39].

1.1.2 Pathogenesis

Similar to all bunyaviruses, with the exception of those in the Hantavirus genus, Rift Valley fever virus replicates in arthropods[40]. The mosquito's gut initially becomes infected and virus becomes detectable in the saliva after several days or weeks. When the vector feasts on the blood meal of a vertebrate, their viremic saliva enters into the lymphatic system or capillaries of the host. The primary site of replication has yet to be elucidated, but the most probable sites are the regional lymph nodes[41].

The virus has a broad tropism and is known to infect peripheral blood mononuclear cells (PBMCs), including monocytes and macrophages, lymphocytes, and dendritic cells. RVFV can also target other organs, such as the liver and brain[42]. Although full characterization of cell surface attachment factors has not been determined, heparin sulfate has been implicated in facilitating attachment and entry into cells[43].

RVFV epizootics are typically characterized by sweeping abortion storms ruminants, with nearly 100% of pregnant animals miscarrying. Loss of the fetus is characterized by cytokine storms, multiple organ infection, and fetal necrosis. Neonatal sheep that are less than one month old are also extremely susceptible to infection. After a short incubation period of 12 to 24 hours, a marked fever develops in the animal with progression to death occurring within 24 to 72 hours post-infection due to necrotic hepatitis[44]. Ninety to 100% of sheep neonates succumb to clinical disease. Neonatal calves are less susceptible to RVFV infection, but disease

course is similar and mortality estimates still range from 10-70%. Adult livestock are more resistant to the virus, but death still occurs in 10-30% and 5-10% of infected sheep and cattle, respectively. Symptoms of Rift Valley fever include fever, lethargy, anorexia, diarrhea, and nasal discharge. In highly susceptible animals, death results from hemorrhage and hepatitis[45].

In humans, the spectrum of disease ranges from mild or inapparent to severe outcomes that result in death. The incubation period ranges from 2 to 6 days, typically followed by a self-limiting illness characterized by fever, anorexia, myalgia, headache, and gastrointestinal distress. For the majority of cases, the disease fully resolves itself in several weeks with no sequelae. However, in a small percentage of those infected (1-2%), the infection can progress to more severe clinical disease including hemorrhagic fever, acute hepatitis, encephalitis, and retinitis leading to blindness. In those most severely affected, coagulopathy, multiple organ dysfunction, renal and hepatic failure, and meningoencephilitis precipitate death. Approximately 10-20% of those with a severe infection will have a fatal outcome. The mechanisms of pathogenesis that determine clinical disease outcome are not fully understood and are still under investigation[46].

1.1.3 Host immune response

Detailed studies of the immune response elicited by RVFV infection are severely lacking. Due to the inadequacy of infrastructure and resources in many endemic countries and the absence of well-characterized animal models, research has been limited. Like most ssRNA viruses, RVFV encodes virulence factors to defend against the immune system by inhibiting the actions of type I interferon[47] and deregulating a broad spectrum of cytokines[48].

Although innate components of immunity have undergone cursory investigation, information on the humoral response during severe infection is extremely sparse. It is understood that neutralizing antibodies are directed at the viral glycoproteins, Gn and Gc, and that high titers are thought to mediate initial and persistent protection against disease[49]. Studies using passive antibody transfer have proven inconclusive[63]. Binding antibodies are targeted against the N protein, the dominant immunogen in bunyavirus infection[50], and are responsible for promoting complement fixation[51]. Antibodies are also raised against the non-structural NSs protein, but to a lesser degree. It is relatively unknown what responsibilities these antibodies play in influencing clinical outcome. Huge gaps exist in our knowledge of the humoral immune response to RVFV, and further examination of all aspects of immunity is needed.

1.1.4 Prevention and Treatment

At this time, no licensed vaccines[52] or therapeutics[53] exist for Rift Valley fever virus. Several live-attenuated strains of Rift have been tested for their prophylactic efficacy, all of which conferred varying degrees of protection and possessed specific limitations.

The first vaccine created, known as the Smithburn strain, was adapted by serial passaging of virulent RVFV in mouse brain. It induced abortions and teratogenesis in livestock and was reserved for use with non-pregnant animals only during devastating outbreaks. Since then, a formalin-inactivated Smithburn vaccine has been produced, but is not as efficacious as its live-attenuated counterpart and requires several boosters. Reversion to virulence is a risk for the mouse-adapted strain[54].

In response to the 1977 Egyptian outbreak, USAMRIID developed a vaccine strain by passaging the virulent ZH548 strain 12 times in the presence of 5-fluorouracil to induce mutagenesis[55]. This strain was designated MP-12, and contained mutations in all 3 genome segments[56]. The vaccine initially showed promise in evoking immunogenicity to RVFV, but after a trial in South Africa, it was revealed that MP-12 could induce abortion and teratogenesis during early pregnancy in sheep. Despite this setback, MP-12 is still being pursued for human and veterinary prophylaxis[57].

The last existing vaccine is Clone 13, an isolate of RVFV found to contain a large deletion in the coding region for the NSs protein, the major virulence factor of RVFV[58]. This deletion makes it impossible for the virus to revert to wildtype, and trials in multiple species of livestock have shown impressive antibody responses. Importantly, immunization did not induce abortion in pregnant animals, nor did it result in negative side effects[59]. Although none of the vaccines are FDA approved, some countries still utilize them to inoculate livestock during periods of outbreak[60].

No therapeutics exist for established RVFV infection, other than palliative care[61]. Ribavirin has been tested as an antiviral treatment for experimentally-infected animals with minimal success. The compound does not efficiently cross the blood-brain barrier and would be of limited use in preventing delayed-onset encephalitis. Astonishingly, RVFV-infected laboratory animals receiving ribavirin treatment were associated with a pronounced shift in disease characteristics from acute hepatitis to neurological disease and hence, the drug is not recommended for those suffering from mild cases of disease[62]. Alternative treatments such as interferon and passive antibody therapy have been pursued, but efficacy from these clinical trials has proven to be inconclusive[63].

1.2 PUBLIC HEALTH SIGNIFICANCE OF RVFV INFECTION

Due to the potential for severe consequences during disease outbreaks, Rift Valley fever virus is classified as a category A overlap select agent by the Centers for Disease Control and Prevention (CDC), the National Institute for Allergy and Infectious Diseases (NIAID), and the USDA. It is also categorized as a high-consequence pathogen with the potential for international spread (List A) by the World Health Organization (WHO) for Animal Health[64]. Because of this, RVFV is considered a potential bioterror agent that could have dramatic direct and indirect impact in countries that are currently free of the virus. Due to the increase in international travel and trade in the past decades, transmitting RVFV into naïve regions through these means is also highly feasible[65].

1.2.1 Biological warfare

Intentional exposure to Rift Valley fever virus through bioterrorism is a definite possibility, especially due to certain viral characteristics. The selection of viruses for use as bioweapons depends on several factors: the relative infectivity in the target populations, the ability to induce a fatal or incapacitating illness, the ease of propagating large amounts of high titer stock in cell culture, the competency to be transmitted via aerosol, and the dearth of measures available to control the virus. Several viruses in the Bunyaviridae and Togaviridae families have been cited as potentially important bioterrorism weapons, with Rift Valley fever virus holding the highest priority[66].

RVFV was heavily researched during the Cold War for use as a weaponized aerosol. The aerosol form proved to be very stable, and could facilitate infection through inhalation. At room temperature with 30% relative humidity, RVFV was found to have a half-life of 77 minutes when in aerosol form[67]. During an assessment of the possible damage that a bioterrorist attack with RVFV could cause, consultants from WHO calculated that if 50 kg of virus was released from an aircraft in the center of a population of 500,000 persons, an estimated 35,000 people would be incapacitated and 400 deaths could occur[68]. In epidemic regions human infection rates can reach as high as 35%, as seen from previous outbreaks[69]. Aside from the direct impact on the population, susceptible livestock could be infected and endemic disease could be established in the area[70].

1.2.2 Importation of virus to virgin territories.

As seen with the rapid spread of West Nile virus after its introduction to North America in 1999, there is high potential for foreign viruses to become easily established in the United States. Globalization of commerce and travel has provided likely routes for arbovirus establishment, with high-temperature areas, dense regions of livestock, and places with a bounty of capable mosquito vectors being the most probable regions for the virus to lay its foundation. Although human infection is an immense concern, it has been estimated that the livestock industry could lose billions of dollars due to death of animals and imposed trade restrictions if an outbreak RVFV were to occur in the USA, having severe socio-economic impact on our country[71].

While investigating the potential for RVFV to enter the United States, a pathways analysis revealed that one of the most likely routes of entry is through infected persons or mosquitos arriving by airplane into the country. It was found that over 97% of individuals travelling to the USA from RVFV-endemic countries arrived at airports in one of six population-dense locations: New York City, Washington DC, Atlanta, Baltimore, Newark, and Houston[72]. These areas, as well as several other states, have been identified as particularly vulnerable regions to RVFV exposure.

Other factors come into play for the importation of virus, such as the unknown impact that global warming could have on arbovirus spread[73].

1.3 RVFV ANIMAL MODELS

Animal models are invaluable tools for biomedical research. The models allow for the study of disease pathogenesis, as well as for evaluation of potential prophylaxis and treatment, when human clinical trials are not possible.

Several models including sheep, hamsters, mice, rats, and non-human primates have been used for experimental infections and pathogenesis studies. Choosing the most relevant animal model is based on many factors such as the virus strain, inoculation route and dose, animal age and species, and pathology that is similar to that seen in the natural host. Despite vigorous research efforts, a well-characterized animal model for aerosolized RVFV pathogenesis has not yet been established.

1.3.1 Rats

A study conducted in 1982 by Peters and Sloan revealed that disease outcome was dependent on the strain of inbred rat when inoculated subcutaneously. Wistar-Furth rats became systemically viremic and all succumbed within a few days to acute hepatitis after exposure to the ZH501 strain of RVFV. August Copenhagan Irish (ACI) rats were moderately susceptible to RVFV subcutaneous (s.c.) infection, with half of the rats expiring after displaying neurological symptoms and mimicking the encephalitic disease seen in humans. Lewis rats displayed no clinical disease after s.c. exposure to ZH501 but had detectable viremia and antibody responses, indicating that they were not resistant to infection, but were able to sufficiently control it[74].

The Hartman lab is actively working to establish a rat model for aerosol infection of RVFV, using the ZH501 strain. Similar to the results seen by Peters and Sloan, Wistar-Furth and ACI rats exposed to aerosolized RVFV exhibited extensive hepatic disease and meningoencephilitis, respectively. Mortality rates were 100% for both rat strains when exposed to extremely lose doses of virus, many folds lower than those used in the Peters and Sloan studies. Surprisingly, Lewis rats were not able to efficiently control respiratory infection, and developed lethal neurological disease like that of the ACI rats. Lewis rats were also inoculated s.c., and later re-challenged via aerosol with the identical virus strain to determine if the s.c. infection protected them against aerosol infection. It was found that the prior infection offered no safeguard against aerosol challenge[75], suggesting possible implications for the development and efficacy of vaccines and therapeutics to protect against respiratory exposure.

1.3.2 Non-human primates

Non-human primates (NHPs) are often the most desired animal model when investigating human disease due to similarities in clinical illness. Unlike inbred rat strains, studies with non-human primates have shown more variability in the clinical outcome after RVFV infection. The first NHP study in 1931 described an infection of rhesus macaques that induced a fever response with leukopenia, but did not cause severe disease and death. Different routes of inoculation, such as intraperitoneal (i.p.), intracerebral (i.c.), s.c, and intranasal (i.n.), were attempted to induce a fatal infection, but no clinical symptoms developed. Other studies have found that rhesus macaques and cynomolgus macaques are more susceptible to aerosol RVFV exposure, but low levels of morbitity still persisted[76].

Although rhesus macaques dependably mimic the spectrum of disease seen in humans, studies have shown that less than 20% of the animals develop fatal disease, which is not ideal for the testing and development of pharmaceuticals. Other NHP studies have employed South American capuchins and marmosets as models for infection and found their susceptibility to be slightly higher than other NHP species[77].

Several laboratories are working on the development of a susceptible NHP model that imitates the severe outcomes of human infection including acute hepatitis, delayed-onset encephalitis, and hemorrhagic syndrome. At this time, it appears that the New World common marmoset may represent the most realistic model of the severe clinical outcomes seen in humans. A recent study found the mortality rate for marmosets to range from 25-100%, depending on the route of exposure, with 50-100% of the NHPs displaying clinical symptoms of infection.

Interestingly, marmosets that were inoculated intranasally had the highest rates of morbidity and mortality[78].

Given that aerosol exposure has occurred in laboratory and epidemic settings and is the most likely route of exposure if a bioterrorist attack were to occur, the Hartman lab is currently investigating the pathogenesis of respiratory infection in non-human primates.

2.0 STATEMENT OF THE PROJECT AND SPECIFIC AIMS

Defining an animal model includes characterizing the immune response to the delivered pathogen. The humoral response plays an important role in clearing infections and affecting the pathogenesis of the disease. The goal of this project is to elucidate the total IgG antibody response for the aerosolized rat strains and the total IgG and neutralizing antibody response for the aerosolized non-human primate species that mimic severe clinical manifestations in humans. Determining if the antibody response is responsible for protecting against fatal infection will give insight into possible mechanisms for developing prophylaxis and treatment. *Our working hypothesis is that the humoral response elicited by aerosolized Rift Valley fever virus helps determine the disease outcome for the animal models.* This hypothesis will be tested with the following specific aims.

Aim 1: To develop methodology for measuring antibody responses using an indirect IgG enzyme-linked immunosorbant assay (ELISA) and plaque reduction neutralization test ($PRNT_{50}$) for quantitative determination of antibodies in 3 strains of rats and 4 species of non-human primates exposed to aerosolized Rift Valley fever virus. To address this aim, indirect IgG ELISA and $PRNT_{50}$ protocols were optimized for sensitivity and specificity for use with rat and non-human primate serum samples. The ELISA assay measured the total amount of

RVFV-specific IgG present in the serum of both rats and non-human primates. The $PRNT_{50}$ assay determined the titer of virus-specific neutralizing antibodies present in rat and non-human primate serum.

Aim 2: To characterize the strength and timing of the total IgG antibody response in the 3 rat strains exposed to aerosolized RVFV. Total IgG antibody titers in rat serum were quantitated by indirect ELISA using a sumOD approach. Results were compared between the rats that survived aerosol challenge and those that succumbed to disease. For serial sacrifice studies, the timing of the total IgG antibody response was determined by indirect ELISA using the sumOD approach.

Aim 3: To characterize the strength and timing of both the total IgG and neutralizing antibody responses in 4 species of non-human primate exposed to aerosolized RVFV. Total IgG antibody titers were quantitated in non-human primates by indirect ELISA using a sumOD approach. Results were compared between monkeys that survived aerosol exposure and those that succumbed to disease. Neutralizing antibody titers were measured by a plaque-reduction neutralization test ($PRNT_{50}$) in non-human primate serum samples at various time points to determine their importance in clinical outcome. Pre-infection, intermediate, and endpoint samples were compared between animals, if available.

3.0 MATERIALS AND METHODS

3.1 BIOSAFETY

All experiments using live ZH501 RVFV were performed in the biosafety level 3+ Regional Biocontainment Laboratory (RBL) at the University of Pittsburgh. Powered air purifying respirators (PAPRs) were worn for respiratory protection and all work was conducted in a class II biosafety cabinet using Vesphene IIse (diluted 1:128, Steris Corporation, cat. #646101) as a disinfectant. Work involving MP-12 RVFV was performed under BSL-2 conditions in a class II biosafety cabinet, using Vesphene IIse (1:128)

3.2 CELL CULTURE

Vero E6 and BHK-21 cells (ATCC) were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine (complete DMEM). Cells were split upon reaching confluency using 0.05% trypsin/EDTA. Cultured cells were maintained in a humidified incubator ($37^{o}C$, 5% CO_2).

3.3 GENERATION OF LYSATES

BHK-21 cells were seeded into 4 T-150 flasks using complete DMEM until confluent. Media was removed from confluent flasks. Two flasks received an inoculum of complete DMEM (mock-infected) and two flasks received MP-12 RVFV (kind gift from T. Ross), at an MOI of 0.1. The infected flasks were incubated for 1 hour at 37^{O}C, 5% CO_2 with gentle rocking every 15 minutes. After incubation, the inoculum was removed from the flasks and the cells were washed briefly with DPBS. Thirty mL of Opti-MEM (reduced serum media) supplemented with 1% pen/strep was added to all flasks. The infected flasks were incubated for 2 days and then moved overnight into a -80^{o}C freezer after marked cytopathic effects (CPE) were seen. The flasks were then removed from the freezer and placed back into the incubator to quickly thaw and lyse the cells. After the freeze-thaw cycle, the infected media was centrifuged at 1000 RPM for 10 minutes at 24^{o}C. Lysate was collected and corresponding mock- and MP-12-infected flasks were combined together. A small aliquot from the MP-12-infected flasks was saved for plaque assay titer confirmation. All remaining lysate was inactivated with 0.1% β-propriolactone for 24 hours before moving to the -80^{o}C freezer.

3.4 PLAQUE ASSAY

Vero E6 cells suspended in complete DMEM were seeded into 6-well plates overnight until 95% confluent. Serial dilutions of the sample lysate were prepared in DMEM supplemented with 2% FBS. Media was removed from the cells and 200 µl of the diluted lysate

was added to the wells in duplicate. Plates were incubated for 1 hour with gentle rocking every 15 minutes. After incubation, the inoculum was removed from the wells and nutrient overlay (1X minimum essential medium, 2% FBS, 1% pen/strep, HEPES buffer, and 0.8% SeaKem agarose) was added. After the overlay solidified, the plates were allowed to incubate for 3 days at 37^{o}C, 5% CO_2. After formation of plaques, 37% formaldehyde was added to the wells and allowed to fix in the biosafety cabinet for several hours. Formaldehyde was drained from the plates and disposed of appropriately, and the agar plugs were removed from the wells. Crystal violet working solution (diluted in 35% ethanol) was then added to the plates to visualize the plaques and determine a titer.

3.5 INDIRECT IGG ELISA

Immulon 2HB 96-well plates (Fisher Scientific, cat. #14-245-61) were coated with 100 µl of mock-infected or MP-12-infected inactivated lysate (diluted 1:20 in DPBS) and incubated overnight at 4^{o}C. After washing the plates 3 times with PBS-T (1X PBS + 0.05% Tween, Fluka, cat. #08057-12TAB-F), 200 µL of blocking buffer (PBS-T + 5% blotting grade blocker, Bio-Rad, cat. #170-6404XTU) was added to the wells and allowed to incubate for 1 hour at 37^{o}C, 5% CO_2. Plates were washed 3 times as before, and 100 µL of control or test sera (diluted 1:100-1:6400, four-fold, in blocking buffer) was added in duplicate to the wells. The plates were incubated at 37^{o}C for 1 hour. Plates were washed 3 times, followed by the addition of 100 uL of horseradish peroxidase-conjugated goat anti-rat IgG (H+L, KPL, cat. #14-16-06) or goat anti-monkey IgG (γ, KPL, cat. #074-11-021), diluted 1:4000 in blocking buffer. The secondary

antibody was incubated for 1 hour at 37°C, washed 3 times, and 100 µL of 2,2'-azinodiethylbenzothiazoline sulfonic acid (ABTS) substrate (KPL, cat. # 50-66-18) was added to each well. Plates were incubated in the dark at 37°C for 30 minutes. After that time, 100 µL of ABTS stop solution (1% sodium dodecyl sulfate, KPL, cat. #50-85-01) was added to the wells and the optical density (OD) was determined at 405 nm.

3.6 PLAQUE REDUCTION NEUTRALIZATION TEST

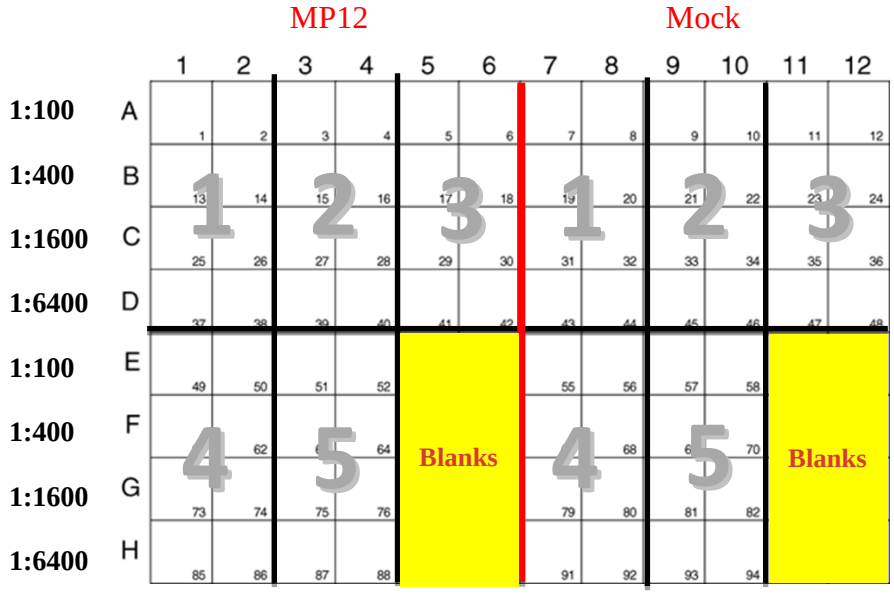
Sera samples to be tested were heat inactivated at 56°C for 30 minutes and then diluted two-fold (1:20-1:40,960) in DMEM + 5% FBS. An equal volume of 100 plaque-forming units (PFU)/0.1 mL of ZH501 RVFV diluted in DMEM + 5% FBS was added to the sera dilutions, making the final dilutions 1:40-1:81,920, two-fold. The virus/serum mixtures were incubated at 4°C overnight and then used to inoculate confluent monolayers of Vero E6 cells in duplicate on 12-well plates. The cells were incubated at 37°C and 5% CO_2 for 1 hour with gentle mixing every 15 minutes. The inoculate was removed completely from the wells and replaced with nutrient overlay (1X minimum essential medium, 2% FBS, 1% pen/strep, HEPES buffer, and 0.8% SeaKem agarose). The plates were incubated for 3 days, after which the cells were fixed with 37% formaldehyde for several hours in the biosafety cabinet. The formaldehyde was drained and the agar overlay was removed before staining with crystal violet working solution for visualization of the plaques. The neutralizing antibody titer was considered positive at the highest serum dilution that inhibited greater than 50% of the plaques as compared to the titration of the virus with no serum.

4.0 RESULTS

4.1 AIM 1: TO DEVELOP METHODOLOGY FOR MEASURING ANTIBODY RESPONSES USING AN INDIRECT IGG ENZYME-LINKED IMMUNOSORBANT ASSAY (ELISA) AND PLAQUE REDUCTION NEUTRALIZATION TEST ($PRNT_{50}$) FOR QUANTITATIVE DETERMINATION OF ANTIBODIES IN 3 STRAINS OF RATS AND 4 SPECIES OF NON-HUMAN PRIMATES EXPOSED TO AEROSOLIZED RIFT VALLEY FEVER VIRUS.

Commercially-produced RVFV antibody detection kits, and even purified antigen for use in these tests, are publically unavailable for scientific purposes. Due to the recent spread of RVFV into virgin territories and its potential for use as a bioweapon, a high-quality, sensitive, specific and safe assay is warranted for diagnostic and research use. RVFV diagnosis is currently accomplished by several techniques, including virus isolation, antigen detection, nucleic acid amplification, and detection of specific antibodies. Unfortunately, these methods tend to be time-consuming and expensive, making rapid diagnosis difficult. Most endemic regions do not have the facilities and resources to safely handle the virus, putting laboratory workers at risk for infection[79]. Several enzyme-linked immunosorbant assays (ELISAs) have been described, although most of these tests are based on reagents that are expensive and onerous to produce[80].

Paweska et al. has previously described and validated methods for an indirect ELISA to measure the IgG response in exposed populations. Using his methodology and a protocol received from USAMRIID (United States Army Medical Research Institute of Infectious Diseases, kind gift from D. Smith) as a guide, we have developed an indirect IgG ELISA using inactivated MP-12-infected lysate as a coating antigen. The antigen is relatively quick, easy, and inexpensive to produce and can be generated in large quantities to ensure a standardized stock. To account for background and reduced specificity, a mock-infected lysate was used for comparison. To determine the strength of the antibody response, we employed a sumOD approach that has been utilized in past studies and is accepted as a standard way of quantifying humoral immunity. SumOD was calculated by measuring the absorbance of multiple serum dilutions in both mock- and MP-12-infected lysate-coated wells. The average optical density from each mock-coated dilution was subtracted from the average optical density of the corresponding MP-12-coated dilution. The differences of the dilutions were added together to obtain the sumOD value (Figure 1). Samples were considered positive for IgG if the sumOD value was greater than the average of the control sumOD plus 3 standard deviations. The control sumODs were calculated using mock-infected control animal samples and were species- and strain-specific for comparison to infected animals. The sumOD provides a more accurate representation of antibody response than a single dilution.



	1	2	3	4	5	6	7	8	9	10	11	12
A	0.121	0.055	0.681	0.751	0.904	0.862	0.077	0.067	0.09	0.081	0.083	0.082
B	0.048	0.048	0.65	0.658	0.785	0.771	0.091	0.049	0.11	0.084	0.075	0.066
C	0.047	0.05	0.486	0.44	0.476	0.506	0.055	0.057	0.072	0.101	0.182	0.09
D	0.052	0.071	0.26	0.255	0.308	0.294	0.078	0.087	0.11	0.079	0.062	0.038
E	0.886	1.137	0.903	0.878	0.132	0.132	0.149	0.235	0.184	0.208	0.163	0.154
F	1.011	1.135	0.941	0.879	0.176	0.169	0.178	0.181	0.162	0.172	0.163	0.154
G	0.678	0.722	0.655	0.669	0.182	0.227	0.215	0.188	0.188	0.188	0.186	0.194
H	0.492	0.455	0.438	0.435	0.175	0.179	0.172	0.183	0.187	0.174	0.184	0.179

	MP12 average						**Mock average**				
	R3A1	R3F1	R3F2	R3G1	R3G2		R3A1	R3F1	R3F2	R3G1	R3G2
1:100	0.088	0.716	0.883	1.0115	0.8905	**1:100**	0.072	0.0855	0.0825	0.192	0.196
1:400	0.048	0.654	0.778	1.073	0.91	**1:400**	0.07	0.097	0.0705	0.1795	0.167
1:1600	0.0485	0.463	0.491	0.7	0.662	**1:1600**	0.056	0.0865	0.136	0.2015	0.188
1:6400	0.0615	0.2575	0.301	0.4735	0.4365	**1:6400**	0.0825	0.0945	0.05	0.1775	0.1805

SumOD (MP12 sum of dilutions - Mock sum of dilutions)						**MP12 average - Mock average**				
	R3A1	R3F1	R3F2	R3G1	R3G2		R3A1	R3F1	R3F2	R3G1	R3G2
1:100-1:6400	-0.0345	1.727	2.114	2.5075	2.1675	**1:100**	0.016	0.6305	0.8005	0.8195	0.6945
						1:400	-0.022	0.557	0.7075	0.8935	0.743
MP12 blank average			**Mock blank average**			**1:1600**	-0.0075	0.3765	0.355	0.4985	0.474
0.19075			0.18575			**1:6400**	-0.021	0.163	0.251	0.296	0.256

Figure 1: 96 well plate set-up and sumOD calculations.

Schematic showing how each plate was arranged, using 5 serum dilution samples and blanks (top). Depiction of the sumOD calculations (bottom). Areas shaded blue represent a mock-infected control animal.

The gold standard for RVFV diagnosis is a plaque reduction neutralization test (PRNT). Although the test is lengthy and requires 5 days for completion, it is highly accurate, can be used for any species, and can validate the results of other assays[81].

Several investigators have previously described protocols for a RVFV $PRNT_{50}$ assay. Based on these methods and guidance from USAMRIID, we have optimized a $PRNT_{50}$ assay for use with ZH501 and our rat and non-human primate serum samples (see Materials and Methods section). This assay was used for validation purposes for our IgG ELISA, as well as to determine the role of neutralizing antibodies in RVFV respiratory infection of our animal models. During infection with RVFV, neutralizing antibodies are raised against the viral glycoproteins and are thought to mediate protection of uninfected cells[82]. There is a dearth of information regarding humoral responses to RVFV and further investigation is warranted to determine the role that neutralizing antibodies play in host immunity.

Using mock-infected control animals as antibody-negative samples and known ZH501-infected animals (based off of fever response and weight loss data, not shown) as antibody-positive samples, we were able to conclude that our indirect IgG ELISA and $PRNT_{50}$ assays are sensitive and specific for RVFV antibodies in both rats and non-human primates.

4.2 AIM 2: TO CHARACTERIZE THE STRENGTH AND TIMING OF THE TOTAL IGG ANTIBODY RESPONSE IN THE 3 RAT STRAINS EXPOSED TO AEROSOLIZED RVFV.

To establish the role that total IgG antibodies play in clinical disease outcome in rats exposed to aerosolized RVFV, we analyzed serum samples collected from Wistar-Furth, ACI, and Lewis rats used in median lethal dose and serial sacrifice studies. Median lethal dose studies were conducted to determine the LD_{50} for aerosolized RVFV, as well as to confirm the disease outcome (hepatic disease, meningoencephilitis, and febrile illness for Wistar-Furth, ACI, and Lewis rats, respectively) for each rat strain, based off of a historical subcutaneous inoculation study by Peters and Sloan. Aerosol exposure to RVFV resulted in acute hepatitis for Wistar-Furth rats and neurological disease for ACI rats, as expected. Surprisingly, respiratory exposure of Lewis rats, which were shown to develop only febrile disease to s.c. infection, mimicked the outcome seen in ACI rats, leading to fatal encephalitis. This suggests that the route of inoculation is a critical determinant of disease outcome, and could result in grave consequences to exposed populations if RVFV were to be weaponized. The median lethal dose was determined to be 2 PFU, 123 PFU, and 112 PFU for Wistar-Furth, ACI, and Lewis rats, respectively (Figure 2). Aerosolization with RVFV caused 100% mortality at much lower doses than was seen through s.c. infection (Table 1).

Table 1: Comparison of disease outcome of different rat strains after subcutaneous or aerosol exposure to RVFV

Rat strain	Dose (pfu)	Subcutaneous*		Aerosol		Comparison S.C. vs. Aerosol
		Ave % mortality	Time to death (days)	Ave % mortality	Time to death (days)	
Wistar-Furth	10^3	90	3	100	4	Severe hepatic disease; similar time frame and clinical signs
ACI	10^3	10	15	100	6	Neurological disease; similar clinical signs; shorter time frame and increased lethality by aerosol
Lewis	10^3	0	N/A	100	7	High lethality at modest doses by aerosol

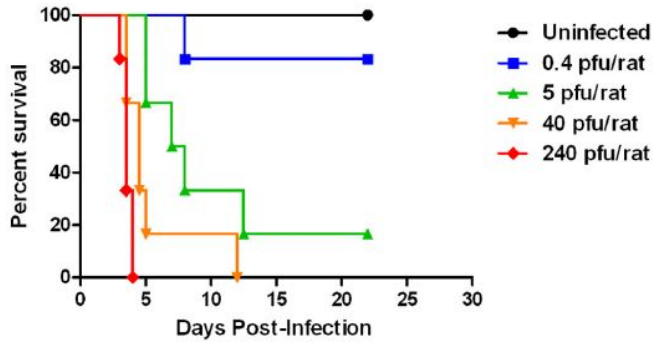
*Subcutaneous exposure data from Peters and Slone (1982).

The clinical outcome results of RVFV infection is influenced by route of administration and rat strain.

To determine the robustness of the IgG antibody response to aerosol RVFV infection, serum samples taken at necropsy from the LD_{50} studies were assayed using our indirect IgG ELISA (Figure 2). For hepatitis-sensitive Wistar-Furth rats, doses above 5 PFU resulted in 100% mortality with sumODs for deceased rats being slightly lower than the sumODs of the survivors at doses of 0.4 and 5 PFU. Wistar-Furth rats were found to be highly susceptible to aerosolized RVFV, with even the lowest dose of less than 1 PFU resulting in fatal hepatic necrosis for one animal. ACI rats, which exhibit the delayed-onset encephalitis seen in humans, were somewhat more resistant to fatal infection. Survival was observed for all dose groups except at the highest dose of 3,900 PFU, although mortality occurred at as low as 20 PFU. Surviving ACI rats displayed a stronger IgG response than the Wistar-Furth and Lewis rats, with

the average sumOD becoming greater as the presented dose increased. Lewis rats, previously shown to control s.c. infection, were found to be vulnerable to neurological disease when subjected to RVFV aerosolization. Complete seroconversion was seen for surviving rats that received doses of 1.5 or 30 PFU, but not all rats that succumbed to infection mounted an IgG response.

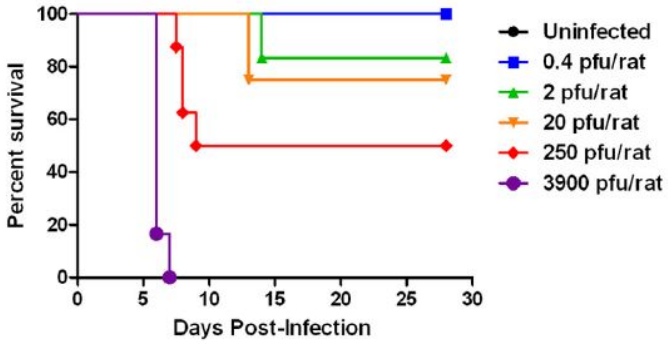
A



Survivors **Deceased**

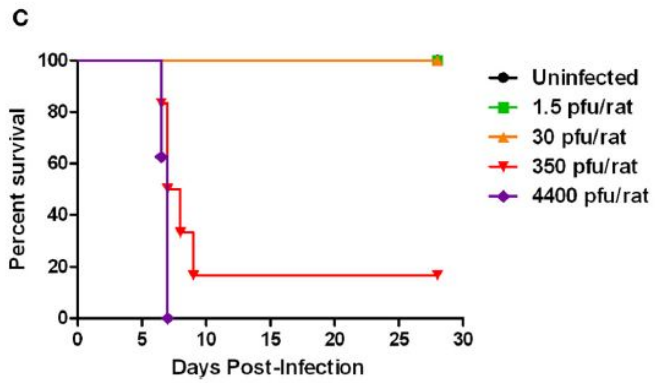
Presented Dose	# of IgG+ rats	Total # of rats	% of IgG+ rats	SumOD Average	# of IgG+ rats	Total # of rats	% of IgG+ rats	SumOD Average	Avg. # days to death
Control	0	6	0%	-0.033	-	-	-	-	-
0.4 PFU	2	4	50%	0.473	0	1	0%	0.112	8
5 PFU	1	1	100%	0.881	1	1	100%	0.348	8
40 PFU	-	-	-	-	NS	NS	NS	NS	NS
240 PFU	-	-	-	-	0	3	0%	0.093	3.67

B



Survivors **Deceased**

Presented Dose	# of IgG+ rats	Total # of rats	% of IgG+ rats	SumOD Average	# of IgG+ rats	Total # of rats	% of IgG+ rats	SumOD Average	Avg. # days to death
Control	0	4	0%	-0.002	-	-	-	-	-
0.4 PFU	0	4	0%	-0.003	-	-	-	-	-
2 PFU	5	5	100%	2.169	-	-	-	-	-
20 PFU	5	5	100%	2.918	2	2	100%	1.373	13
250 PFU	4	4	100%	3.722	2	2	100%	0.312	8
3900 PFU	-	-	-	-	NS	NS	NS	NS	NS



	Survivors				Deceased				
Presented Dose	# of IgG+ rats	Total # of rats	% of IgG+ rats	SumOD Average	# of IgG+ rats	Total # of rats	% of IgG+ rats	SumOD Average	Avg. # days to death
Control	0	5	0%	0.055	-	-	-	-	-
1.5 PFU	5	5	100%	1.790	-	-	-	-	-
30 PFU	3	3	100%	1.586	-	-	-	-	-
350 PFU	NS	NS	NS	NS	1	2	50%	0.136	7.5
4400 PFU	-	-	-	-	4	5	80%	0.234	7

Figure 2: Determination of LD_{50} and IgG antibody response in surviving and deceased rat strains following aerosol exposure to RVFV at various presented doses.

Cohorts of female Wistar-Furth, ACI, and Lewis rats (8-10 weeks old) were challenged with increasing doses of aerosolized RVFV or sham inoculation to determine the median lethal dose (LD_{50}). The rats were monitored daily after exposure for clinical signs, increased temperature, and weight loss. Rats found moribund were euthanized. (A) Wistar-Furth, (B) ACI, and (C) Lewis rats. NS, no serum available for analysis. It should be noted that the total number of rats *listed* for each dose does not always sum to the total number of rats *exposed* to that dose due to unavailability of serum.

For our median lethal dose studies, we have shown that clinical disease outcome and IgG antibody response is dependent on the selection of inbred rat strain. Surviving rats demonstrated complete seroconversion at presented doses above 1 PFU with a higher dose typically correlating with a faster time to death than seen with a lower dose. SumODs, on average, increased as the presented dose of RVFV increased for survivors.

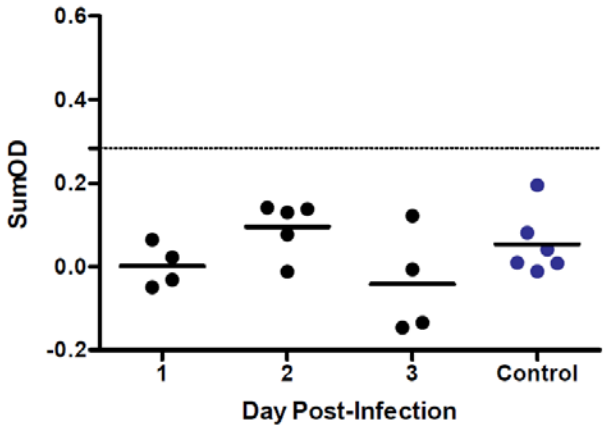


Figure 3: Wistar-Furth rats do not mount a detectable IgG antibody response after respiratory infection with RVFV.

Experimental animals received an aerosolized dose of 400 PFU of ZH501, with control animals receiving a sham aerosol inoculation. Each point represents the IgG sumOD of an individual rat at sacrifice. Control animals were used to determine the cut-off value, which is indicated by the dotted line. Animals were considered positive for IgG if the sumOD was greater than the value of the cut-off.

After comparing IgG titers between surviving and deceased rats, we wanted to determine the emergence and strength of the IgG response in serial sacrifice studies with the 3 strains of rats. To elucidate the timing of the total IgG antibody response, we again utilized our indirect ELISA with a sumOD approach. Since the average time to death was 4 days for Wistar-Furth rats, all animals were presented with a lethal dose of 400 PFU with cohorts sacrificed on days 1, 2, and 3 post-infection. None of the sacrificed animals displayed a detectable IgG response to infection (Figure 3). ACI rats were determined to survive an average of 6 days after aerosol infection. Therefore, cohorts of rats were presented with a fatal dose of 7,500 PFU and were sacrificed on days 1 through 7 post-infection. Complete seroconversion of a cohort appeared on day 6 post-infection (Figure 4), indicating that B cell activation and efficient isotype switching

has occurred by this point. The highest sumODs were seen on day 7 post-infection. Lewis rats, which exhibited a similar clinical disease course as ACI rats in the median lethal dose study, averaged 7 days to death post-infection. Lewis rats were exposed to a deadly dose of 30,000 PFU of RVFV and sacrificed on days 1 through 7. Akin to the ACI rats, Lewis rats also showed complete seroconversion by day 6 post-infection, with the most robust IgG response detected on day 7 post-infection (Figure 5).

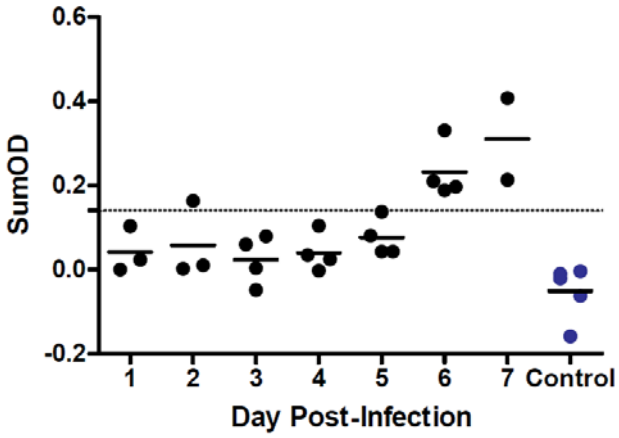


Figure 4: ACI rats develop an IgG antibody response by day 6 post-infection when challenged with aerosolized RVFV.

ACI rats were presented with a dose of 7,500 PFU of ZH501 or were sham-inoculated (control animals). Complete IgG seroconversion occurred by day 6 post-infection. Each point is indicative of the IgG sumOD of a single rat at the time of sacrifice. The dotted line represents the cut-off value, which was determined by control animal sumODs. Rats were deemed positive for IgG if the sumOD exceeded the value of the cut-off.

In conclusion for the serial sacrifice studies, Wistar-Furth rats never mounted a detectable IgG response and likely succumb to infection prior to class switching. ACI and Lewis rats showed total seroconversion by day 6 post-infection, with day 7 showing an even greater antibody response.

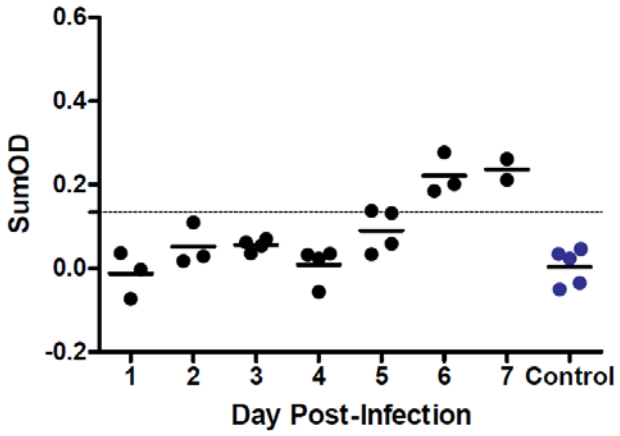


Figure 5: Lewis rats display a similar clinical outcome and IgG antibody response in comparison to ACI rats after aerosol exposure to RVFV.

Lewis rats were delivered a dose of 30,000 PFU of ZH501 by aerosol inoculation. Control rats were sham-inoculated. IgG seroconversion resulted by day 6 post-infection, with the highest average sumODs seen on day 7. Each point denotes the sumOD for an individual animal. The cut-off value, determined by the control rat sumODs, is represented by the dotted line. Animals were considered IgG-positive if the sumOD surpassed the cut-off value.

4.3 AIM 3: TO CHARACTERIZE THE STRENGTH AND TIMING OF BOTH THE TOTAL IGG AND NEUTRALIZING ANTIBODY RESPONSES IN 4 SPECIES OF NON-HUMAN PRIMATE EXPOSED TO AEROSOLIZED RVFV.

A susceptible non-human primate (NHP) model for RVFV aerosol infection has not been established for use in biomedical research[83]. Multiple NHP species from around the world have been evaluated for susceptibility to RVFV through various exposure routes including subcutaneous, intravenous, intraperitoneal, intranasal, and aerosol[84]. Viremia, fever response, and leukopenia are frequently reported in these studies, but most species are resistant to developing severe clinical disease[85]. The rhesus macaque is considered to be an appropriate model to mimic general human infection after i.v. challenge with ZH501. Most animals develop a mild febrile disease, with a lesser proportion progressing to a more critical outcome. As rhesus macaques are only moderately susceptible to infection, they do not provide an ideal model when testing the efficacy of vaccines and therapeutics. Furthermore, i.v. exposure to RVFV is not a natural route of infection, since mosquitoes transmit virus extravascularly[86].

Although non-human primates do not seem to produce a uniform response to RVFV inoculation, previous studies have suggested that the respiratory route of infection may be slightly more pathogenic[87]. To determine which NHP species could best mimic the manifestations seen in severe human disease after respiratory challenge, rhesus macaques, cynomolgus macaques, and African Green monkeys (AGMs) were exposed to high doses (5 $\log_{10}$ PFU/ml) of aerosolized ZH501 in duplicate. A pre-infection blood draw confirmed that the animals were RVFV-naïve by our indirect IgG ELISA and PRNT assays. The NHPs were monitored daily for signs of clinical disease, and implanted telemetry devices were used to

measure physiological responses to infection. Blood samples were taken at the midpoint of the study, with euthanasia and necropsy occurring for the surviving animals at 28 days post-infection. All animals survived to the end of the study, with the exception of an African Green monkey succumbing to neurological disease at day 11 post-infection.

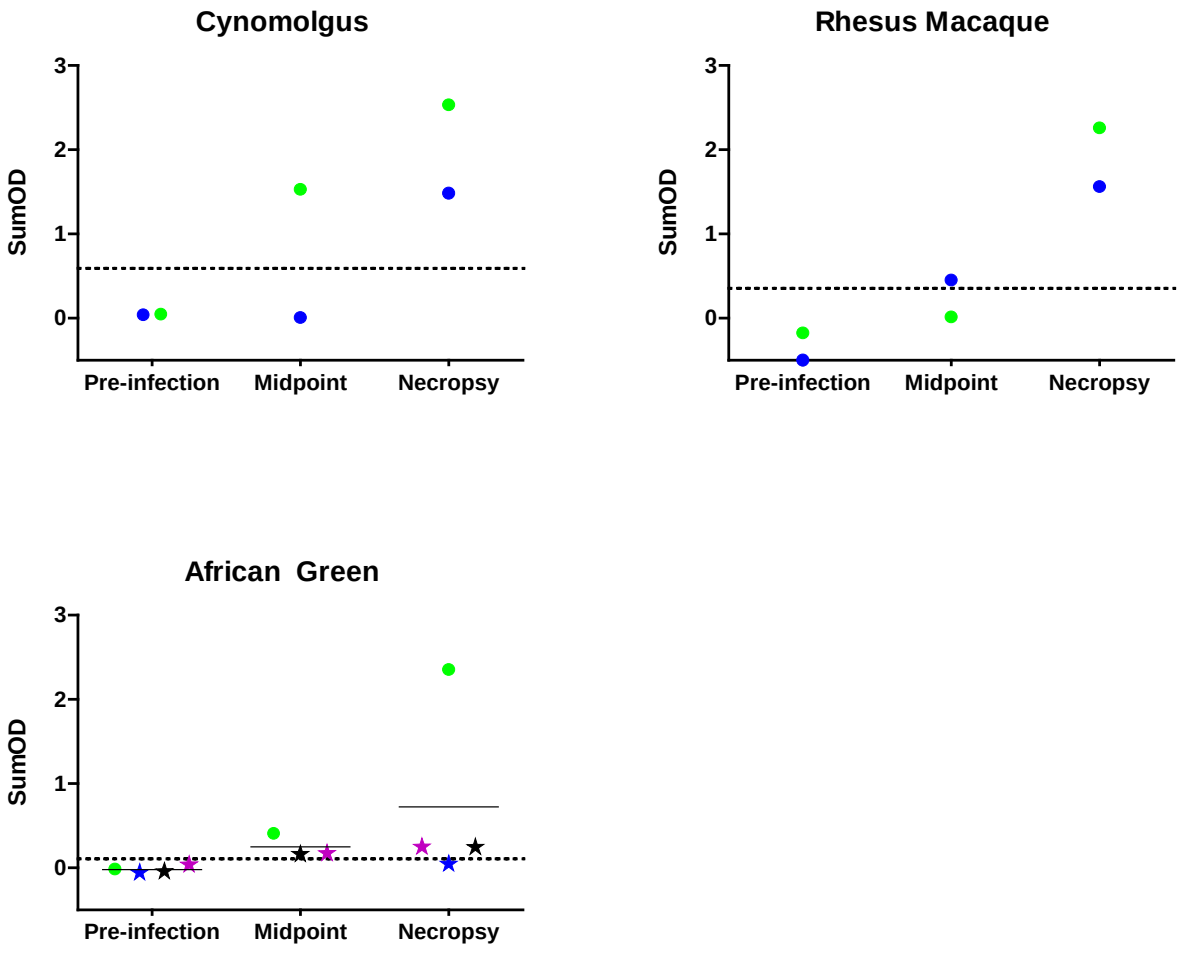


Figure 6: The total IgG responses over time of 3 species of non-human primates exposed to aerosolized RVFV.

Cynomolgus macaques, Rhesus macaques, and African Green monkeys were challenged with high doses of aerosolized ZH501. Blood samples were taken prior to infection, at the midpoint of the study, and at necropsy. Individua l animals are represented by a single color per graph. Blue and green points indicate the first 2 animals of a species infected via aerosol. Stars represent animals that succumbed to infection. The cut-off values, indicated by the dotted line, were determined by the sumODs from the pre-infection bleed. Animals were considered IgG-positive if the sumOD value exceeded that of the cut-off.

Since the AGM species showed promise of a susceptible model, a cohort of an additional 2 AGMs was challenged with comparable doses of aerosolized RVFV. Both of these animals developed symptoms of the same neurological disease seen in the AGM with the fatal infection, and ultimately were euthanized 11 days post-exposure. This species will be further evaluated in future studies for their likelihood to be used as a realistic model of severe neurological infection. Blood samples taken at pre-infection, midpoint, and necropsy were assayed using our indirect ELISA to determine the strength of the IgG response to respiratory exposure to RVFV (Figure 6). All surviving NHPs showed a robust antibody response at necropsy, with the general trend of an increasing sumOD at each subsequent time point after infection. Not all surviving animals developed a strong IgG response by the midpoint of the study, but extremely high neutralizing antibody titers were detected in both the midpoint and necropsy samples by $PRNT_{50}$ for all NHPs (Table 2). The AGM that developed the fatal infection did not seroconvert to IgG, but did mount a vigorous neutralizing antibody response with a titer of 1:5,120. All surviving animals, however, had neutralizing titers greater than 1:81,920.

Table 2: Overview of non-human primate aerosol exposure study

				Pre-infection		Midpoint		Necropsy	
Animal ID	Species	Presented Dose (PFU)	Day Euthanized	Average SumOD	$PRNT_{50}$	Average SumOD	$PRNT_{50}$	Average SumOD	$PRNT_{50}$
M5-12	Cyno	1.1×10^5	Survivor	0.084	1:40	0.033	>1:81920	2.402	>1:81920
M6-12	Cyno	1.9×10^5	Survivor	0.019	<1:40	2.332	>1:81920	3.651	>1:81920
M7-12	Rhesus	1.1×10^5	Survivor	-0.693	<1:40	0.549	>1:81920	1.857	>1:81920
M8-12	Rhesus	4.7×10^5	Survivor	-0.490	<1:40	0.049	1:20480	2.940	>1:81920
M10-12	AGM	2.5×10^5	11	-0.111	<1:40	N/A	N/A	0.147	1:5120
M11-12	AGM	6.7×10^4	Survivor	-0.154	<1:40	0.834	>1:81920	3.665	>1:81920
M81-12	AGM	7.3×10^5	11	0.039	1:40	0.164	>1:81920	0.245	>1:81920
M82-12	AGM	5.6×10^5	11	-0.044	1:40	0.174	>1:81920	0.248	>1:81920

Two animals of different non-human primate species (cynomolgus macaques, rhesus macaques, and African Green monkeys) were exposed to aerosolized RVFV after a pre-infection bleed. The primates were monitored daily for signs of illness and weight loss, and implanted telemetry devices recorded temperature changes. Blood was drawn at the midpoint at the study, and taken again at necropsy. Serum samples were used in indirect ELISA assays and plaque reduction neutralization tests to determine the total IgG response and neutralizing antibody titers, respectively, at midpoint and necropsy. Text color refers to results in Figure 5.

Recently, a study reported that the common marmoset was a more useful model of severe RVFV infection than other NHP species[88]. The marmosets in this study were challenged via i.v., s.c., or i.n. exposure routes, and it was discovered that animals inoculated intranasally developed the highest rates of morbidity and mortality. To determine if marmosets could be a useful model for aerosol infection with RVFV, a cohort of 2 animals were exposed to aerosolized RVFV. After infection, telemetry implants monitored physiological parameters, and the animals were evaluated daily for signs of disease. Ten days after aerosol exposure, both marmosets began showing neurological signs and were thereafter euthanized and necropsied.

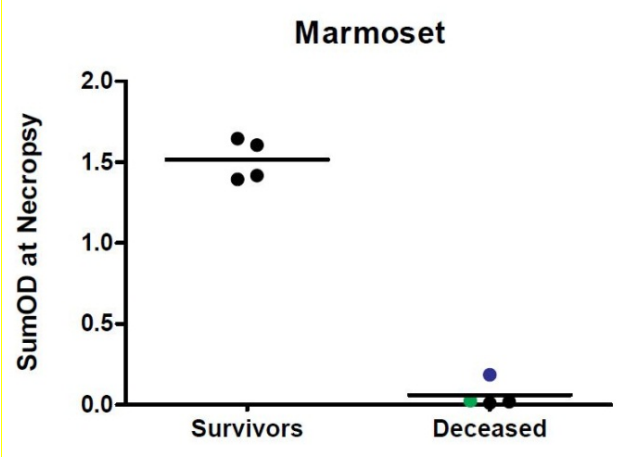


Figure 7: Comparison of sumOD values at necropsy in surviving and deceased marmosets.

A total of 8 common marmosets were aerosol-challenged with RVFV. Four animals displayed neurological symptoms at days 9 and 10 and were euthanized. The results obtained from the first 2 animals used in the study are depicted by blue and green dots.

To establish if the results were reproducible and the species was susceptible to respiratory RVFV exposure, we infected 6 additional marmosets by aerosol at increasing doses of $\log_{10}$. Two marmosets developed lethal encephalitis on days 9 and 10, respectively, and were euthanized. The 4 remaining marmosets survived until the end of the study. Samples taken at necropsy were used to determine sumODs and neutralizing antibody titers using our developed IgG ELISA and PRNT assays (Figure 7, Table 3). Surviving animals had markedly higher average sumODs than the deceased animals, with all animals displaying extremely high neutralizing antibody titers. Although the animal that succumbed on day 9 post-infection had a neutralizing titer of 1:40,960, it did not prove effective in conferring protection against disease.

Table 3: Overview of the humoral immune responses seen in marmosets after respiratory infection with ZH501

Survivors				Deceased				
Animal ID	Presented Dose (PFU)	Average SumOD	$PRNT_{50}$	Animal ID	Presented Dose (PFU)	Average SumOD	$PRNT_{50}$	Day Euthanized
M183-12	9.1×10^{1}	1.646	>1:81920	M88-12	2.4×10^{3}	0.185	>1:81920	10
M184-12	6.0×10^{1}	1.607	>1:81920	M89-12	3.6×10^{4}	0.026	>1:81920	10
M185-12	4.2×10^{2}	1.418	>1:81920	M186-12	2.1×10^{3}	0.011	NS	10
M188-12	1.5×10^{4}	1.394	>1:81920	M187-12	1.5×10^{5}	0.020	1:40960	9

Eight common marmosets were aerosol-challenged with varying doses of RVFV. Results are divided by those surviving infection, and those that did not. Blue and green text refers to the first 2 animals challenged in the study. NS, no serum available for analysis.

5.0 DISCUSSION

Rift Valley fever virus is an important zoonotic arbovirus, responsible for causing severe outbreaks of disease in livestock and humans in Africa and the Arabian Peninsula. Due to certain viral characteristics such as ability to be aerosolized, availability of competent transmission vectors, and globalization of travel and trade, RVFV has high potential to be introduced intentionally or accidently to virgin territories, leading to dramatic socio-economic consequences. Since RVFV is a category A select agent with concern about its potential use as an aerosol, understanding the pathogenesis of inhalational disease is a top priority for the development of medical countermeasures and defense against potential biological warfare.

Cases of Rift Valley Fever resulting from inhalational exposure have been reported in laboratory settings, but due to the lack of public health infrastructure in most endemic countries, few cases of respiratory RVFV infection have been confirmed during outbreaks. It is unknown whether the route of exposure determines or influences the outcome of human clinical disease. Because human trials are unethical and likely implausible during epidemics, well-defined animal models must be developed for research purposes.

Defining an animal model of includes characterizing the host immune response to the delivered pathogen. The humoral response plays an important role in clearing infections and affecting the clinical course of the disease. Determining what role the antibody response plays in

protecting against fatal infection will give insight into possible mechanisms for developing vaccines and therapeutics. There is worry that aerosolized RVFV will be used as a biological weapon, which dictates the urgent need for countermeasures.

Based on the rat model described by Peters and Sloan in 1982, s.c. infection of Wistar-Furth, ACI, and Lewis rats with the ZH501 strain of RVFV resulted in necrotic hepatitis, meningoencephilitis, and a mild febrile illness, respectively. As each inbred rat strain modeled one of the distinct outcomes seen in human RVFV infection, it was suggested that host genetic susceptibility determined the clinical course of disease[89]. In a medial lethal dose study, we have shown that exposure to low or moderate doses of aerosolized RVFV results in acute hepatic disease for Wistar-Furth rats and neurological disease for both ACI and Lewis rats.

To investigate the role of the humoral response in shaping disease outcome, rates of IgG seroconversion and sumOD values were compared between the surviving and deceased rats at corresponding presented doses. Aerosol exposure to RVFV causes higher mortality rates and faster time to death in inbred rats compared to subcutaneous infection, even at low to moderate doses. For all rats, a presented aerosol dose above 1 PFU resulted in complete seroconversion for surviving animals. This suggests that inhalation of a single virion can lead to infection and that a population's exposure to even a small amount of aerosolized RVFV could have dire repercussions.

Wistar-Furth rats, which quickly progressed to acute hepatitis after aerosol infection, reached 100% mortality at extremely low doses of ZH501. When comparing average sumOD values between surviving and deceased rats at the two lowest doses, survivors had at least twice the average sumOD than deceased rats that received the same dose. The average time to death for the deceased rats was 8 days, which should have allowed sufficient time for the rats to

seroconvert to IgG. Therefore, it is possible that the higher IgG antibody titers seen for the survivors conferred protection against fatal disease. Overall, Wistar-Furth rats had lower sumOD averages than the other inbred rat strains.

Several factors may have positively or negatively affected the calculated average sumOD for the Wistar-Furth rats. Of the surviving rats that were presented a dose of 0.4 PFU, only half of the exposed seroconverted, indicating that some rats may not have been infected by such a low dose. Due to unavailability of serum, not every rat was included in the sumOD average, and several averages were determined by only one animal, which would not be representative of the cohort. Since Wistar-Furth rats fully succumbed to the highest doses of RVFV, a low presented dose may not have had the ability to elicit a strong IgG response, with higher sumODs being attainable by exposure to median doses of virus. The fastest time to death and lowest average sumOD were seen in rats that received an inoculation of the highest dose. Because rats were euthanized at days 3 and 4 at this dose, it is likely that they did not have sufficient time to mount an IgG response. We demonstrated that these rats do not mount a detectable IgG response by day 3 post-infection. The Wistar-Furth model of aerosol challenge faithfully represents the severity of RVFV-induced hepatitis in humans. As infected hosts do not have adequate time to mount a specific and robust antibody response before fatal hepatic disease sets in, demand for an immunogenic vaccine to prevent infection is more apparent than the deficit of post-exposure treatment for this clinical outcome.

A proportion of ACI rats were shown to develop neurological disease after s.c. infection with RVFV, mimicking the delayed-onset encephalitis recognized in severe human cases. After an aerosol challenge to determine the median lethal dose, exposed rats displayed the same neurologic symptoms as seen with s.c. infection. Similar to Wistar-Furth rats, ACI rats

succumbed faster and at lower doses than required by s.c. inoculation. Surviving ACI rats yielding the highest average sumODs seen in the study.

Rats exposed to median doses of RVFV resulted in both survival and death. Of those that succumbed, the average time to death was 13 days post-infection. Even at nearly two weeks after exposure, the IgG response for deceased rats was less than half of that of the survivors, based on average sumOD. As demonstrated by the Wistar-Furth and Lewis rats, the sumOD average of the deceased ACI rats infected with moderate doses should have been robust enough to protect against fatal disease. Because antibodies are too large to cross the blood-brain barrier[90], it is possible that the IgG response could have protected against death if the virus did not breach the CNS. There may also be a certain threshold that antibody titers must cross before protection can occur.

In surviving rats challenged with higher doses of RVFV, average sumODs were nearly 12 times higher than the sumODs of the deceased at a comparable dose. We observed the emergence of the IgG antibody response at day 6 post-infection for this strain, albeit at weak levels. Although the deceased rats fully seroconverted by day 8 post-infection, the average sumODs remainined low, suggesting that a robust IgG response potentially lessens disease severity and decrease mortality rates.

When inoculated s.c. with RVFV, Lewis rats proved able to control the virus and resist disease. Interestingly, Lewis rats were unexpectedly susceptible to respiratory RVFV infection and displayed neurological symptoms similar to those exhibited by ACI rats. As the Lewis rat data conflicted with prior reports, a cohort of rats was administered a comparable s.c. dose of virus. All rats survived s.c. infection without showing signs of illness (data not shown). This finding suggests that the route of infection can influence clinical outcome, and that aerosol

exposure to RVFV may be more burdensome than originally conceived from a biodefense standpoint. A possible explanation for the encephalitis seen in Lewis rats is that the virus infiltrates the central nervous system (CNS) through the olfactory bulb, but mechanisms for the discrepancy between the route of infection and clinical outcome seen in the Lewis strain warrants further investigation.

Even at the highest presented doses of RVFV, complete seroconversion of a dose cohort did not occur by day 7 post-infection, with several rats deemed IgG-negative. Our Lewis serial sacrifice data indicates that the IgG immune response appears 6 days post-challenge, with sumODs increasing slightly on day 7, analogous to ACI rats. The lack of seroconversion could indicate a delayed humoral response to aerosol infection and antagonism of the innate immunity in this rat strain.

Our rat studies have shown that RVFV challenge via aerosol results in higher morbidity and mortality when compared to other inoculation routes. At presented doses above 1 PFU, total IgG seroconversion occurred for all surviving rats, indicating extremely high infectivity of aerosolized RVFV. These characteristics of respiratory infection make aerosolized RVFV a serious threat from a military perspective, and further justify the pressing need for vaccine and therapeutic development. Selection of inbred rat strain significantly impacts the disease course, although outcome can vary based upon the route of infection, as demonstrated by our Lewis rat studies.

The varied IgG responses seen between the different rat strains and how they correlate with presented dose could create obstacles for the development of vaccines, since immunization would need to protect against all clinical forms of inhalational disease. For example, protective antibodies cannot cross the blood-brain barrier, so the mechanism for protection against

neurological disease would need to be directed at eliciting both cell-mediated and humoral responses. As hepatic disease is characterized by localized regions of necrosis, a vaccine eliciting a powerful killer T-cell response could exacerbate pathogenicity. Since we are investigating inoculation with RVFV at lung (and possibly olfactory) mucosa, prophylaxis geared toward evoking a strongly neutralizing IgA response could be most effective in preventing all forms of clinical disease.

Non-human primates are commonly considered to be one of the most realistic models to study the pathogenesis of human disease due to shared similarities in presentation of clinical illness. Unlike inbred rats, NHPs have shown inconsistencies in disease outcome after infection with RVFV. While most NHPs do develop viremia after challenge, they often remain asymptomatic[91]. As the ultimate goal is to develop and test medical countermeasures for severe disease, a highly susceptible and well-defined NHP model is desired. Several different Old World and New World species have been infected with RVFV with unpromising results. Although the rhesus macaque mimics the human spectrum of disease with less than 20% of those exposed progressing to critical outcomes after i.v. infection, large cohorts of animals would be needed and the studies would be extremely costly, as well as unnatural. Few NHP studies have utilized aerosolized RVFV and data is lacking for this inoculation route.

Rhesus macaques, cynomolgus macaques, and African Green monkeys were respiratory-challenged with high doses of RVFV. The rhesus and cynomolgus macaques failed to show any clinical signs of disease, despite exhibiting a biphasic fever response (data not shown). Eleven days post-infection, one AGM began displaying symptoms of neurological disease and was euthanized. The moribund animal had previously developed an infection at the site of the telemetry implant. To dispel the possibility that secondary infection contributed to the AGM's

fatal disease, an additional 2 AGMs were aerosol-challenged and were euthanized on day 11 post-infection after showing symptoms of encephalitis.

Based on pre-infection, intermediate, and necropsy bleeds from the 3 NHP species, we determined that the sumOD increased at each time point for survivors. Seroconversion occurred for some of the NHPs by the midpoint bleed, but no correlation was observed between survival and presence of IgG antibodies at midpoint.

Neutralizing antibodies are thought to be protective against RVFV infection, and could possibly have an effect on clinical outcome[92]. By the midpoint of the study, all NHPs had exceptionally high neutralizing antibody titers, but despite the titers, the antibodies did not prove to be neuroprotective for the deceased AGMs. This situation, like that of the encephalitic rats, may also illustrate the effects of antibodies failing to cross the blood-brain barrier. Determining if AGMs are an appropriate model for human neurological disease will be in evaluated in future studies.

Neutralizing antibodies can exist in any combination of the 5 antibody isotypes. Since the IgG sumODs of the euthanized NHPs were very low, it is possible that another isotype, such as IgM, could constitute the majority of the neutralizing titers. Although this isotype constitutes a very small percentage of serum antibodies, IgA antibodies may play an important role in halting the progression of disease resulting from mucosal infection. The importance of individual antibody isotypes must be further examined, and may include measurement of serum IgM levels and determination of IgA titers in bronchoalveolar lavage (BAL) fluid.

Historical studies have indicated that aerosol challenge of NHPs may be marginally more pathogenic than other inoculation routes and a recent study from USAMRIID found common marmosets to be susceptible to intranasal infection. To this end, we challenged a pair of

marmosets through inhalational exposure to ZH501. At 10 days post-infection, both marmosets were discovered moribund with encephilitis and were subsequently euthanized. Six additional marmosets were added to the study and received low, moderate, and high doses of RVFV. Two marmosets displaying neurologic symptoms were euthanized on days 9 and 10 post-challenge, with the remaining monkeys surviving to the end of the study.

Serum samples taken from each animal at necropsy revealed that surviving marmosets had high average IgG sumODs and exceptionally potent neutralizing titers. Average sumODs for deceased animals were barely detectable, but neutralizing antibody titers were as strong as the titers for survivors. As this phenomenon was recognized across multiple NHP species and rat strains after respiratory exposure to RVFV, continued exploration of the humoral immune response and determining the role, if any, that neutralizing antibodies play in neurologic disease progression are necessary to gain insight into NHP pathogenesis.

A possibility to address the discordance between neutralizing and IgG antibodies lays in the target of the antibodies. As previously described, our PRNT50 assay measures the neutralizing antibodies present in serum, which are directed at the viral glycoproteins. The neutralizing antibodies bind to the glycoproteins and therefore prohibit infection from occurring. IgG antibody titers were quantitated with our IgG ELISA using whole lysate to coat the plates. Because whole lysate is used in place of a purified protein in our assay, detectable IgG antibodies can be directed at any viral protein, including glycoproteins, the N protein, NSs, and others. Neutralizing antibodies did not seem to play an important role in limiting disease progression, indicating that antibodies to non-structural proteins (such as the immunodominant N protein) may confer greater protection against disease. Further investigation is needed to

elucidate the mechanisms and roles of antibodies targeted to structural and non-structural proteins.

Examining innate immune factors, such as cytokines and interferon, and adaptive components, like CD8+ T cells, will further our understanding of the complex relationship between aerosol infection and host immunity, and will contribute to the development of well-defined rat and NHP animal models. Considering that the introduction of RVFV to naïve regions is a genuine possibility, understanding the immunology of the disease is imperative for the development of efficacious vaccines and effective treatments. A greater scope of knowledge will hopefully provide insight to the underlying mechanisms of disease progression, viral or host characteristics responsible for triggering severe outcomes in certain individuals, and the means by which clinical disease can occur in the presence of high neutralizing antibody titers.

BIBLIOGRAPHY

1. Ikegami, T. & Makino, S. The Pathogenesis of Rift Valley Fever. *Viruses* **3**, 493–519 (2011).

2. Bird, B. H., Ksiazek, T. G., Nichol, S. T. & Maclachlan, N. J. Rift Valley fever virus. *J. Am. Vet. Med. Assoc.* **234**, 883–893 (2009).

3. Boshra, H., Lorenzo, G., Busquets, N. & Brun, A. Rift Valley Fever: Recent Insights into Pathogenesis and Prevention. *Journal of Virology* **85**, 6098–6105 (2011).

4. Pepin, M., Bouloy, M., Bird, B. H., Kemp, A. & Paweska, J. Rift Valley fever virus(Bunyaviridae: Phlebovirus): an update on pathogenesis, molecular epidemiology, vectors, diagnostics and prevention. *Vet. Res.* **41**, 61 (2010).

5. Paweska, J. T., Burt, F. J. & Swanepoel, R. Validation of IgG-sandwich and IgM-capture ELISA for the detection of antibody to Rift Valley fever virus in humans. *J. Virol. Methods* **124**, 173–181 (2005).

6. Narayanan, A. *et al.* Curcumin inhibits Rift Valley fever virus replication in human cells. *J. Biol. Chem.* **287**, 33198–33214 (2012).

7. Coetzer, J. A. The pathology of Rift Valley fever. I. Lesions occurring in natural cases in new-born lambs. *Onderstepoort J. Vet. Res.* **44**, 205–211 (1977).

8. Ross, T. M., Bhardwaj, N., Bissel, S. J., Hartman, A. L. & Smith, D. R. Animal models of Rift Valley fever virus infection. *Virus Research* **163**, 417–423 (2012).

9. Smith, D. R. *et al.* The pathogenesis of Rift Valley fever virus in the mouse model. *Virology* **407**, 256–267 (2010).

10. Balkhy, H. H. & Memish, Z. A. Rift Valley fever: an uninvited zoonosis in the Arabian peninsula. *Int. J. Antimicrob. Agents* **21**, 153–157 (2003).

11. Turell, M. J. *et al.* Vector competence of Kenyan Culex zombaensis and Culex quinquefasciatus mosquitoes for Rift Valley fever virus. *J. Am. Mosq. Control Assoc.* **23**, 378–382 (2007).

12. Sidwell, R. W. & Smee, D. F. Viruses of the Bunya- and Togaviridae families: potential as bioterrorism agents and means of control. *Antiviral Res.* **57**, 101–111 (2003).

13. Bird, B. H., Khristova, M. L., Rollin, P. E., Ksiazek, T. G. & Nichol, S. T. Complete genome analysis of 33 ecologically and biologically diverse Rift Valley fever virus strains reveals widespread virus movement and low genetic diversity due to recent common ancestry. *J. Virol.* **81**, 2805–2816 (2007).

14. Daubney, R., Hudson, J. R. & Garnham, P. C. Enzootic hepatitis or rift valley fever. An undescribed virus disease of sheep cattle and man from east africa. *The Journal of Pathology and Bacteriology* **34**, 545–579 (1931).

15. Jost, C. C. *et al.* Epidemiological assessment of the Rift Valley fever outbreak in Kenya and Tanzania in 2006 and 2007. *Am. J. Trop. Med. Hyg.* **83**, 65–72 (2010).

16. Sindato, C., Karimuribo, E. & Mboera, E. G. The epidemiology and socio-economic impact of Rift Valley fever epidemics in Tanzania: A review. *Onderstepoort J. Vet. Res.* **79**, E1 (2012).

17. Hassan, O. A., Ahlm, C., Sang, R. & Evander, M. The 2007 Rift Valley fever outbreak in Sudan. *PLoS Negl Trop Dis* **5**, e1229 (2011).

18. Hoogstraal, H., Meegan, J. M., Khalil, G. M. & Adham, F. K. The Rift Valley fever epizootic in Egypt 1977-78. 2. Ecological and entomological studies. *Trans. R. Soc. Trop. Med. Hyg.* **73**, 624–629 (1979).

19. El Mamy, A. B. O. *et al.* Unexpected Rift Valley Fever Outbreak, Northern Mauritania. *Emerging Infectious Diseases* **17**, 1894–1896 (2011).

20. Abdo-Salem, S. *et al.* Can environmental and socioeconomic factors explain the recent emergence of Rift Valley fever in Yemen, 2000-2001? *Vector Borne Zoonotic Dis.* **11**, 773–779 (2011).

21. Al-Afaleq, A. I. & Hussein, M. F. The status of Rift Valley fever in animals in Saudi Arabia: a mini review. *Vector Borne Zoonotic Dis.* **11**, 1513–1520 (2011).

22. Andriamandimby, S. F. *et al.* Rift Valley fever during rainy seasons, Madagascar, 2008 and 2009. *Emerging Infect. Dis.* **16**, 963–970 (2010).

23. Bird, B. H. *et al.* Rift valley fever virus lacking the NSs and NSm genes is highly attenuated, confers protective immunity from virulent virus challenge, and allows for differential identification of infected and vaccinated animals. *J. Virol.* **82**, 2681–2691 (2008).

24. Nabeth, P. *et al.* Rift Valley fever outbreak, Mauritania, 1998: seroepidemiologic, virologic, entomologic, and zoologic investigations. *Emerging Infect. Dis.* **7**, 1052–1054 (2001).

25. Wilson, M. L. Rift Valley fever virus ecology and the epidemiology of disease emergence. *Ann. N. Y. Acad. Sci.* **740**, 169–180 (1994).

26. Métras, R. *et al.* Rift Valley fever epidemiology, surveillance, and control: what have models contributed? *Vector Borne Zoonotic Dis.* **11**, 761–771 (2011).

27. Martin, V. *et al.* The impact of climate change on the epidemiology and control of Rift Valley fever. *Rev. - Off. Int. Epizoot.* **27**, 413–426 (2008).

28. Arishi, H. M., Aqeel, A. Y. & Al Hazmi, M. M. Vertical transmission of fatal Rift Valley fever in a newborn. *Ann Trop Paediatr* **26**, 251–253 (2006).

29. Seufi, A. M. & Galal, F. H. Role of Culex and Anopheles mosquito species as potential vectors of rift valley fever virus in Sudan outbreak, 2007. *BMC Infectious Diseases* **10**, 65 (2010).

30. Konrad, S. K. & Miller, S. N. A temperature-limited assessment of the risk of Rift Valley fever transmission and establishment in the continental United States of America. *Geospat Health* **6**, 161–170 (2012).

31. Kakani, S., LaBeaud, A. D. & King, C. H. Planning for Rift Valley fever virus: use of geographical information systems to estimate the human health threat of white-tailed deer (Odocoileus virginianus)-related transmission. *Geospat Health* **5**, 33–43 (2010).

32. Abu-Elyazeed, R. *et al.* Prevalence of anti-Rift-Valley-fever IgM antibody in abattoir workers in the Nile delta during the 1993 outbreak in Egypt. *Bull. World Health Organ.* **74**, 155–158 (1996).

33. SMITHBURN, K. C. & MAHAFFY, A. F. Rift Valley fever; accidental infections among laboratory workers. *J. Immunol.* **62**, 213–227 (1949).

34. Madani, T. A. *et al.* Rift Valley fever epidemic in Saudi Arabia: epidemiological, clinical, and laboratory characteristics. *Clin. Infect. Dis.* **37**, 1084–1092 (2003).

35. Mohamed, M. *et al.* Epidemiologic and clinical aspects of a Rift Valley fever outbreak in humans in Tanzania, 2007. *Am. J. Trop. Med. Hyg.* **83**, 22–27 (2010).

36. Frese, M., Kochs, G., Feldmann, H., Hertkorn, C. & Haller, O. Inhibition of bunyaviruses, phleboviruses, and hantaviruses by human MxA protein. *J. Virol.* **70**, 915–923 (1996).

37. Hartley, D. M., Rinderknecht, J. L., Nipp, T. L., Clarke, N. P. & Snowder, G. D. Potential effects of Rift Valley fever in the United States. *Emerging Infect. Dis.* **17**, e1 (2011).

38. De Boer, S. M. *et al.* Heparan sulfate facilitates Rift Valley fever virus entry into the cell. *J. Virol.* **86**, 13767–13771 (2012).

39. Nfon, C. K., Marszal, P., Zhang, S. & Weingartl, H. M. Innate immune response to Rift Valley fever virus in goats. *PLoS Negl Trop Dis* **6**, e1623 (2012).

40. Weekly epidemiological record. (2008).

41. Bhardwaj, N., Heise, M. T. & Ross, T. M. Vaccination with DNA Plasmids Expressing Gn Coupled to C3d or Alphavirus Replicons Expressing Gn Protects Mice against Rift Valley Fever Virus. *PLoS Neglected Tropical Diseases* **4**, e725 (2010).

42. Head, J. A., Kalveram, B. & Ikegami, T. Functional analysis of Rift Valley fever virus NSs encoding a partial truncation. *PLoS ONE* **7**, e45730 (2012).

43. McElroy, A. K. & Nichol, S. T. Rift Valley fever virus inhibits a pro-inflammatory response in experimentally infected human monocyte derived macrophages and a pro-inflammatory cytokine response may be associated with patient survival during natural infection. *Virology* **422**, 6–12 (2012).

44. Wallace, D. B. *et al.* Protective immune responses induced by different recombinant vaccine regimes to Rift Valley fever. *Vaccine* **24**, 7181–7189 (2006).

45. Fafetine, J. M. *et al.* Cloning and expression of Rift Valley fever virus nucleocapsid (N) protein and evaluation of a N-protein based indirect ELISA for the detection of specific IgG and IgM antibodies in domestic ruminants. *Vet. Microbiol.* **121**, 29–38 (2007).

46. McElroy, A. K., Albariño, C. G. & Nichol, S. T. Development of a RVFV ELISA that can distinguish infected from vaccinated animals. *Virol. J.* **6**, 125 (2009).

47. Gray, K. K. *et al.* Chemotactic and inflammatory responses in the liver and brain are associated with pathogenesis of Rift Valley fever virus infection in the mouse. *PLoS Negl Trop Dis* **6**, e1529 (2012).

48. Vialat, P., Muller, R., Vu, T. H., Prehaud, C. & Bouloy, M. Mapping of the mutations present in the genome of the Rift Valley fever virus attenuated MP12 strain and their putative role in attenuation. *Virus Res.* **52**, 43–50 (1997).

49. Ikegami, T. & Makino, S. Rift Valley fever vaccines. *Vaccine* **27**, D69–D72 (2009).

50. Billecocq, A. *et al.* NSs protein of Rift Valley fever virus blocks interferon production by inhibiting host gene transcription. *J. Virol.* **78**, 9798–9806 (2004).

51. Dungu, B. *et al.* Evaluation of the efficacy and safety of the Rift Valley Fever Clone 13 vaccine in sheep. *Vaccine* **28**, 4581–4587 (2010).

52. Von Teichman, B. *et al.* Safety and efficacy of Rift Valley fever Smithburn and Clone 13 vaccines in calves. *Vaccine* **29**, 5771–5777 (2011).

53. Ikegami, T. Molecular biology and genetic diversity of Rift Valley fever virus. *Antiviral Res.* **95**, 293–310 (2012).

54. *Airborne Survival of Rift Valley Fever Virus*. 11 (USAMRIID, 1982).

55. Christopher, G. W., Cieslak, T. J., Pavlin, J. A. & Eitzen, E. M., Jr Biological warfare. A historical perspective. *JAMA* **278**, 412–417 (1997).

56. Meegan, J., Watten, R. & Laughlin, L. Clinical experience with Rift Valley fever in humans during the 1977 Egyptian epizootic. *Contr. Epidem. Biostatist* 114–123 (1981).

57. Borio, L. *et al.* Hemorrhagic fever viruses as biological weapons: medical and public health management. *JAMA* **287**, 2391–2405 (2002).

58. Gould, E. A. & Higgs, S. Impact of climate change and other factors on emerging arbovirus diseases. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **103**, 109–121 (2009).

59. Peters, C. J. & Slone, T. W. Inbred rat strains mimic the disparate human response to Rift Valley fever virus infection. *J. Med. Virol.* **10**, 45–54 (1982).

60. Bales, J. M., Powell, D. S., Bethel, L. M., Reed, D. S. & Hartman, A. L. Choice of inbred rat strain impacts lethality and disease course after respiratory infection with Rift Valley Fever Virus. *Frontiers in Cellular and Infection Microbiology* **2**, (2012).

61. Findlay, G. The infectivity of Rift Valley fever for monkeys. *Trans. R. Soc. Trop. Med. Hyg.* **XXVI**, 161–168 (1932).

62. Smith, D. R. *et al.* Development of a novel nonhuman primate model for Rift Valley fever. *J. Virol.* **86**, 2109–2120 (2012).

63. Jansen van Vuren, P. & Paweska, J. T. Laboratory safe detection of nucleocapsid protein of Rift Valley fever virus in human and animal specimens by a sandwich ELISA. *J. Virol. Methods* **157**, 15–24 (2009).

64. Jansen van Vuren, P., Potgieter, A. C., Paweska, J. T. & Van Dijk, A. A. Preparation and evaluation of a recombinant Rift Valley fever virus N protein for the detection of IgG and IgM antibodies in humans and animals by indirect ELISA. *J. Virol. Methods* **140**, 106–114 (2007).

65. Paweska, J. T., Jansen van Vuren, P. & Swanepoel, R. Validation of an indirect ELISA based on a recombinant nucleocapsid protein of Rift Valley fever virus for the detection of IgG antibody in humans. *J. Virol. Methods* **146**, 119–124 (2007).

66. Paweska, J. T., Mortimer, E., Leman, P. A. & Swanepoel, R. An inhibition enzyme-linked immunosorbent assay for the detection of antibody to Rift Valley fever virus in humans, domestic and wild ruminants. *J. Virol. Methods* **127**, 10–18 (2005).

67. Swanepoel, R. *et al.* Comparison of techniques for demonstrating antibodies to Rift Valley fever virus. *J Hyg (Lond)* **97**, 317–329 (1986).

68. Heise, M. T. *et al.* An alphavirus replicon-derived candidate vaccine against Rift Valley fever virus. *Epidemiology and Infection* **137**, 1309 (2009).

69. Morrill, J. C. & Peters, C. J. Protection of MP-12-vaccinated rhesus macaques against parenteral and aerosol challenge with virulent rift valley fever virus. *J. Infect. Dis.* **204**, 229–236 (2011).

70. Peters, C. J. *et al.* Experimental Rift Valley fever in rhesus macaques. *Arch. Virol.* **99**, 31–44 (1988).

71. Miller, W., Demchak, P., Rosenberger, C., Dominik, J. & Bradshaw, J. Stability and infectivity of airbourne yellow fever and rift valley fever viruses. *The American Journal of Hygiene* **77**, 114–121 (1963).

72. Campana, V. *et al.* Development of antibody fragments for immunotherapy of prion diseases. *Biochem. J.* **418**, 507–515 (2009).

73. Easterday, B. C.65–119 (University of Wisconsin, 1965).

74. Dodd, K. A., Bird, B. H., Metcalfe, M. G., Nichol, S. T. & Albariño, C. G. Single-dose immunization with virus replicon particles confers rapid robust protection against Rift Valley fever virus challenge. *J. Virol.* **86**, 4204–4212 (2012).