

Identification and evaluation of antivirals for Rift Valley fever virus

by

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B.S., Northeast Agricultural University, 2009

M.S., Northeast Agricultural University, 2012

AN ABSTRACT OF A DISSERTATION

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DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

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Abstract

Rift Valley fever virus (RVFV) is an enveloped, negative-sense, ssRNA virus with a tripartite genome that causes morbidity and mortality in both livestock and humans. Although RVFV is mainly circulating in mainland Africa, this arthropod-borne virus is a potential threat to the other parts of the world. No fully licensed vaccines for human or animal use in the U.S., and effective antiviral drugs have not been identified. As virulent RVFV strains are only handled in biosafety level (BSL) 3 or higher level facilities in the U.S., few laboratories have access to RVFV which limits antiviral development. However, it is crucial to develop effective antivirals to protect public and animal health.

Animal models that reproduce Rift Valley fever are vital to identifying and developing antiviral compounds. The currently available attenuated RVFV strain, MP12, provides a BSL-2 challenge model virus for preliminary investigations of RVFV prior to using the virulent RVFV strains. All strains of RVFV have a highly conserved genome, indicating that antivirals or vaccines effective against any RVFV strain will most likely be effective for all RVFV strains. Therefore, we hypothesize that the MP12 is a suitable model virus that can be used for identification and evaluation of effective RVF antivirals.

The first objective of this project was to establish a mouse model susceptible to MP12 infection. Based on the literature, we selected and screened six different strains of mice to test their susceptibilities to MP12. We found the STAT-1 knockout mice are the most susceptible to MP12 infection based on clinical symptoms, mortality, viremia, virus replication, histopathological, and immunochemical analyses. Importantly, these mice displayed acute-onset hepatitis and delayed-onset encephalitis similar to severe cases of human RVFV infection.

Our second objective was to identify potential antiviral drugs *in vitro*. We developed and employed a cell-based assay using the recombinant MP12 virus expressing *Renilla* luciferase to screen a library of 727 small compounds purchased from National Institutes of Health. Of the compounds, 23 were identified and further tested for their inhibitory activities on the recombinant MP12 virus expressing green fluorescent protein. Further plaque reduction assays confirmed that two compounds inhibited replication of parental RVFV MP12 strain with limited cytotoxic effects. The 50% inhibitory concentrations using an MP12 multiplicity of infection (MOI) of 2 were 211.4 μ M and 139.5 μ M, respectively.

Our third objective was to evaluate these two candidates, 6-azauridine and mitoxantrone, *in vivo* using our mouse model. After one-hour post MP12 infection via an intranasal route, treatment was given intranasally twice daily. Mice treated with placebo and 6-azauridine displayed severe weight loss and reached the threshold for euthanasia with obvious neurological signs, while mice treated with ribavirin (a known antiviral drug) or mitoxantrone showed delayed onset of disease. This result indicates that the mitoxantrone can improve the outcome of RVFV infection in our mouse model.

The underlying mechanism of mitoxantrone to inhibit RVFV replication remains to be investigated. Our studies build the foundation for identification and development of antivirals against RVFV in a BSL-2 environment.

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Chapter 1 - General Introduction

1.1 Rift Valley fever

Rift Valley fever (RVF) is an acute, febrile disease caused by Rift Valley fever virus (RVFV) that affects cattle, buffalo, llamas, alpacas, sheep, goats, camels, and humans. Age and species, among other factors, determine the severity of the disease. In adult cattle and sheep, the fatality rates may reach 30%; however, in younger animals fatality could reach 100% (J. M. Meegan & Bailey, 1988). In humans infected with RVFV, clinical signs include weakness, headache, body ache, fever, and/or photophobia (B. C. Easterday, 1964; Weiss, 1957). Although most patients recover, less than 5% develop complications characterized by hepatitis, retinitis, hemorrhagic fever, and encephalitis with long-term neurologic deficits (Arthur, 2000; Gowen & Holbrook, 2008; Ikegami & Makino, 2011). Since RVFV is threatening the health of both humans and animals, the CDC and the U.S Department of Agriculture (USDA) have classified RVFV as an overlap Select Agent (Bird, Ksiazek, Nichol, & MacLachlan, 2009). Moreover, due to the threat of terrorism, RVFV has been registered as a category A agent in the CDC bioterrorism list. (Bird et al., 2009).

1.1.1 History of RVF

RVF was first identified during an investigation into an epidemic of abortion among sheep along the shores of Lake Naivasha in the Rift Valley of Kenya in 1930 (Daubney, Hudson, & Garnham, 1931). Since then, countries in Africa, such as Niger, Mauritania, Madagascar, Sudan, Kenya, and Egypt, have experienced numerous RVF outbreaks (Figure 1). In the 1950s, the first cases of RVF outside of Kenya appeared in South Africa (Gear, De Meillon, Measroch, Davis, & Harwin, 1951). Subsequently, larger epizootics and epidemics occurred in Egypt in 1977-1978 (JM Meegan, 1979) and Mauritania in the late 1980s during flooding (Digoutte &

Peters, 1989). The 1990's brought the first cases of RVF outside of mainland Africa, occurring on the island of Madagascar. Fortunately, further research suggested the outbreak was caused by repeated introductions of RVFV from mainland Africa and not persistence of the virus in the local environment (Carroll et al., 2011).

In 2000, a pivotal RVF epidemic occurred outside of Africa in Yemen and Saudi Arabia that resulted in significant human fatality. Although both livestock and humans were affected, it was estimated that approximately 200 humans died out of 2,000 hospitalized patients (Balkhy & Memish, 2003; Shoemaker et al., 2002). After this outbreak, in 2006-2007, Somalia, Kenya, and Tanzania, also experienced a RVF outbreak with substantial human deaths. The epidemic resulted in 315 human deaths out of 1,062 cases, along with the loss of livestock (WHO, 2007). The spread of RVFV outside Africa was mainly due to the commercial transport of infected animals and windborne movement of infected mosquitoes. The most recent occurrence of RVF case outside of Africa occurred in 2016 in China (J. Liu et al., 2017). The single infected patient had recently returned from a trip to Angola, Africa, a RVF non-endemic country (J. Liu et al., 2017). In the last decade, the CDC estimates the total mortality rate of hospitalized RVF patients to be around 10%. The potential of dissemination of RVF poses a threat to not only the livestock industry but also to public health (Ahmed et al., 2009; J. Liu et al., 2017).

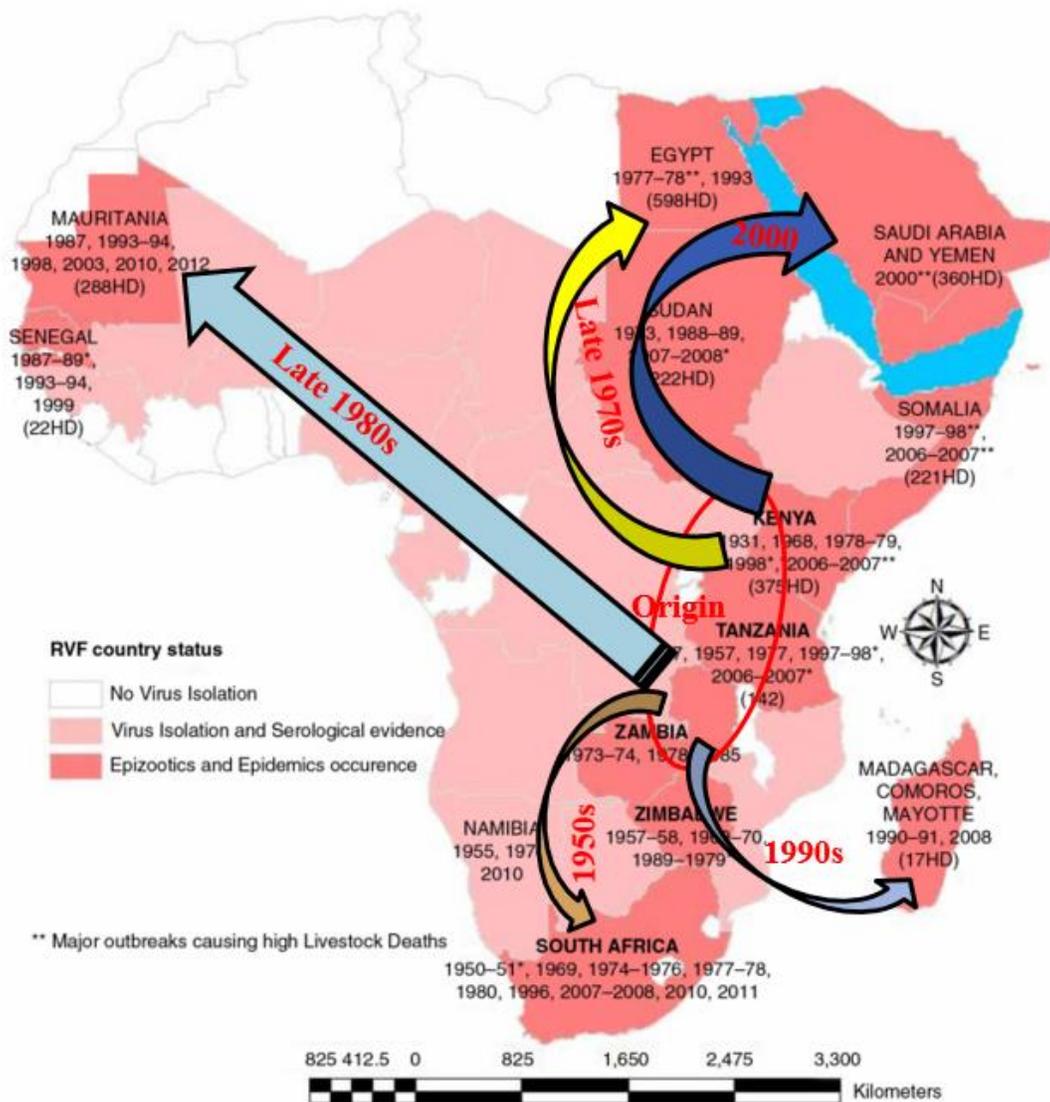


Figure 1 Distribution and spread of RVFV

Countries that have reported substantial epizootics and epidemics are in dark pink. Countries with serologic evidence or virus isolation are marked with light pink. Cases were recognized in South Africa in 1950s. Larger epizootics and epidemics occurred in Egypt in 1978-1979, in Mauritania in the late 1980s, and in Madagascar in the 1990s. And around 2000 in Yemen and Saudi Arabia, RVFV emerged for the first-time outside Africa in the Arabian Peninsula. Adapted from Nanyingi, M. O (2015). A systematic review of Rift Valley Fever epidemiology 1931-2014. *Infect Ecol Epidemiol*, 5, 28024.

1.1.2 RVFV Classification

RVFV is a spherical, negative-sense, single-stranded, three segmented RNA virus which belongs to the genus *Phlebovirus*, in the family of *Phenuiviridae* (D. H. Bishop et al., 1980). The nomenclature system for RVFV includes a geographic origin, the successive isolate number from that location, and the year of isolation, such as RVFBJ01/2016. So far, only one serotype is recognized, but RVFV strains have variable virulence. There are at least seven major genetic lineages, while Kenya-1 and Kenya-2 sub-lineages are predominant recently (M. Pepin, M. Bouloy, B. H. Bird, A. Kemp, & J. Paweska, 2010). However, at the nucleotide and amino acid level, all strains of RVFV remain closely related, which suggests a vaccine or an antiviral drug effective to one RVFV strain should have an effect on all the other RVFV strains.

1.1.3 RVFV Transmission

As an arbovirus, RVFV is thought to be maintained in mosquitoes. Because mosquitoes can maintain RVFV for their whole lives and transmit it to offspring via eggs (Linthicum, Britch, & Anyamba, 2016). In the natural environment, RVF breaks out after periods of heavy rainfall and flooding because a significant amount of RVFV infected mosquito eggs might hatch and transmit the virus to animals and humans (Davies, Linthicum, & James, 1985). Other species of mosquitoes such as *Culex*, *Mansonia*, and *Anopheles* can be mechanical vectors for disease transmission from infected animals to healthy animals or to humans through blood feeding (Turell, Dohm, & Fonseca, 2014). Sandflies, midge, and tick can also serve as vectors based on the published data (Fontenille et al., 1998). Direct contact with infected body fluid or tissues can also result in human and ruminant infection (R. Swanepoel & Coetzer, 2004). Currently, no human-to-human transmission has been reported (Figure 2).

The severity of RVFV infection is varied among animals. Primary hosts of RVFV are cattle, sheep, goats, llamas, alpacas, dromedaries, rodents, wild ruminants, buffaloes, antelopes, wildebeest, etc. Besides transmission by *Aedes* mosquitoes, ruminants can also transmit the virus to their offspring through vertical transmission. Lambs, kids, puppies, kittens, mice, and hamsters are extremely susceptible to RVFV infection with 70–100% mortalities; sheep and calves are highly susceptible, and the mortalities are 20–70%; cattle, goats, African buffalo, domestic buffalo, Asian monkeys, and humans are moderately susceptible; camels, equids, pigs, dogs, cats, African monkeys, baboons, rabbits, and guinea pigs are resistant; birds, reptiles, and amphibians are not susceptible (Committee on Foreign and Emerging Diseases of the United States Animal Health Association, 2008). Humans are susceptible to RVFV infection (major zoonosis), while African monkeys and domestic carnivores present a transitory viremia after infection (Bird et al., 2009).

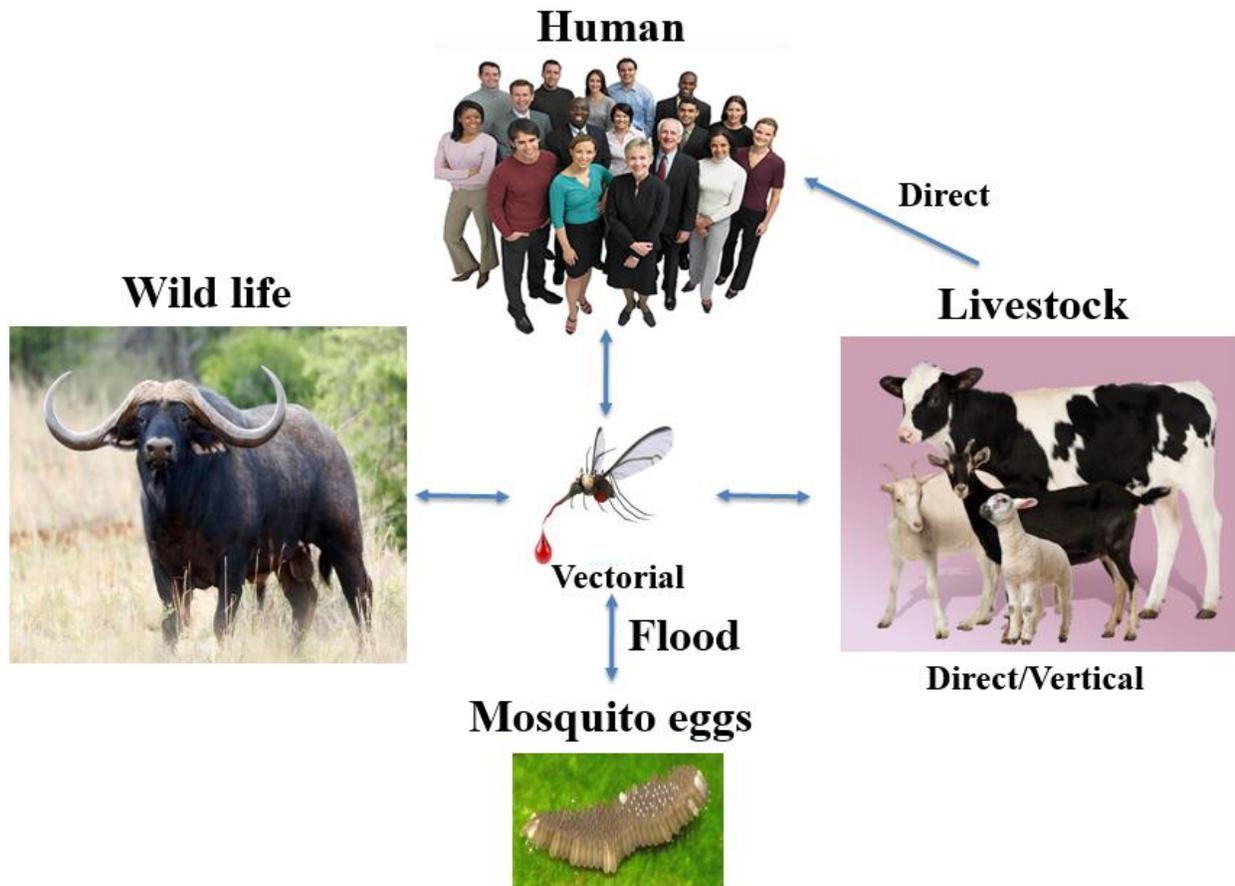


Figure 2. Transmission Cycles of RVFs

Three transmission routes have been identified for RVFV: vectorial, direct, and vertical routes. Mosquitoes play a major role in RVFV transmission because RVFV can be maintained in mosquitoes' eggs which can survive for years. Once heavy rainfalls or warm seasons occurred, the infected mosquitoes' eggs would be stimulated to emerge from the resting stage to adult and start to spread disease among species, subsequently initiate the epizootic outbreaks. The direct transmission has also been identified among ruminant. No case of human-to-human transmission has been identified.

1.1.4 RVFV lifecycle

RVFV can infect and replicate in a variety of cell lines, such as African green monkey Vero cell line, white-tailed deer cell lines, and mosquito cell line (Gaudreault, Indran, Bryant, Richt, & Wilson, 2015; Weingartl, Zhang, et al., 2014). RVFV is usually transmitted by the vectorial route between different hosts. After infection, RVFV replicates at the infection site and the draining lymphatics followed by seeding the target organs via viremia. The main targeted organs of RVFV are liver, brain, spleen, thymus, and pancreas (Gommet et al., 2011; Reed et al., 2012). The lifecycle of RVFV in cells includes three steps: 1. virus attachment, entry, and fusion; 2. genome transcription, translation, and replication; 3. virion assembly, budding, and release (Figure 3) (Walter & Barr, 2011).

Dermal dendritic cells (DCs) are the first cells that the RVFV may encounter when an infected mosquito bites a mammalian host (Švajger, Anderluh, Jeras, & Obermajer, 2010). The glycoproteins of RVFV may interact with the DC specific intercellular adhesion molecule 3 (ICAM-3) grabbing non-integrin (DC-SIGN) which is expressed on DCs and some macrophages (Lozach et al., 2011; Phoenix et al., 2016; Soilleux, Barten, & Trowsdale, 2000). Another receptor, glycosaminoglycan heparan sulfate which is abundantly expressed on the membrane of most animal cells, has been proven to be essential for RVFV to gain entry to cells (De Boer, Kortekaas, de Haan, et al., 2012). As RVFV has a broad range of hosts and cell tropism, it is not surprising to see that RVFV used more than one receptor to facilitate its entry. After the receptor binding, the virion enters the cell via endocytotic route (De Boer, Kortekaas, Spel, et al., 2012; Harmon et al., 2012). Because of histidine protonation occurs in the acidic microenvironment in the endosomal compartments, Gc protein is reorganized subsequently and results in membrane

fusion (De Boer, Kortekaas, Spel, et al., 2012; Dessau & Modis, 2013; Filone, Heise, Doms, & Bertolotti-Ciarlet, 2006; Garry & Garry, 2004).

Next, the viral ribonucleoproteins (vRNPs) are delivered to the cytoplasm in where transcription and replication take place (Bouloy & Weber, 2010). To transcribe the negative stranded vRNAs into mRNAs, the L protein mediates the endonuclease activity to steal the 5' cap from a host mRNA which is used to prime the transcription (Reguera, Weber, & Cusack, 2010). The mRNAs are further truncated at the 3' end relative to the genome template, and a transcription termination signal was observed on the ambisense S segments (Albariño, Bird, & Nichol, 2007; Mir, Duran, Hjelle, Ye, & Panganiban, 2008; Sen & Blau, 2005; Weil et al., 2012). Limited references are published related to the switch mechanism between transcription and replication (Bouloy & Weber, 2010). During replication, the segments replicate via a process involving complementary RNAs (cRNAs). Then the cRNAs serve as templates for the synthesis of new vRNAs. In the mature RVFV virions, a small amount of cRNAs has been identified. Reassortment occurs during virus replication since RVFV is a segmented RNA virus. Whole-genome sequencing followed by phylogenetic analysis of a RVFV isolation in the latest imported human case of RVFV in China revealed a new reassortant from lineage E (L and M segments) and from lineage A (S segment) of RVFV (J. Liu et al., 2017). This fact indicates that the live RVFV vaccines could change some key characteristics, such as virulence and Differentiating Infected from Vaccinated Animals (DIVA) markers, through reassortment with wild-type strains.

Virions assemble at the Golgi and egress by budding in the Golgi compartment followed by exocytosis (Viruses & Fauquet, 2005). The localization of the Gn glycoprotein to the Golgi is critical for virus maturation and assembly (S. Gerrard & S. Nichol, 2002; M. Pepin, M. Bouloy, B. Bird, A. Kemp, & J. Paweska, 2010; Wasmoen, Kakach, & Collett, 1988). Sonja R. et al.

demonstrated that the Gn and Gc heteromeric complex forms in the endoplasmic reticulum (Gerrard & Nichol, 2007). Then the virions assemble at the Golgi and egress by budding into the lumen of the Golgi apparatus (Viruses & Fauquet, 2005). The budding is also observed at the plasma membrane in the rat hepatocytes (G. Anderson & Smith, 1987). Finally, the progeny virions are transported by vacuoles to the plasma membrane and exocytosis takes place (M Pepin et al., 2010; Walter & Barr, 2011).

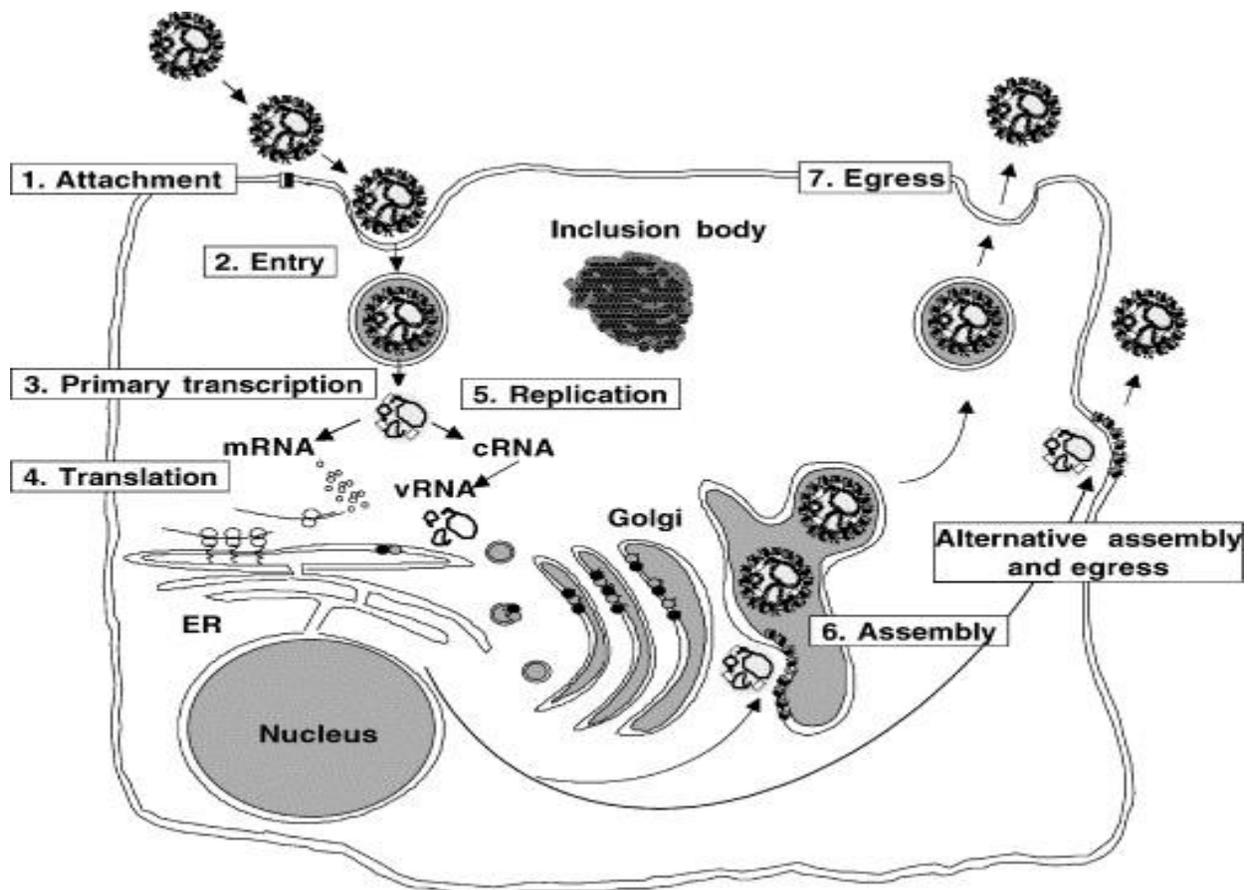


Figure 3. Schematics of RVFV replication cycle

A model of infection cycle for RVFV. Copied from Whitehouse, C. A. (2004). Crimean–Congo hemorrhagic fever. *Antiviral Research*, 64(3), 145-160.

1.1.5 Morphology

RVFV particles have an icosahedral structure with a T=12 triangulation number, ranging from 90–110 nm in diameter (Ellis, Simpson, Stamford, & Abdel Wahab, 1979; Huisken, Overby, Weber, & Grunewald, 2009) and can be observed under 40,000× electron microscopy magnification (Sherman, Freiberg, Holbrook, & Watowich, 2009) (Figure 4A). The virion envelope is composed of a lipid bilayer and two glycoproteins, Gn and Gc which are regularly arranged on its surface forming 5-8 nm length sub-units. Only one related phlebovirus, Uukuniemi virus, is known to have this structure (Freiberg, Sherman, Morais, Holbrook, & Watowich, 2008; Sherman et al., 2009). Inside the envelope, the vRNP corresponding to the S, M, and L segments, numerous copies of the nucleoprotein N, and the RNA-dependent RNA polymerase L are packaged (Figure 4B). Viruses within the *Phenuiviridae* family, they do not have matrix proteins; however, the bridge function of matrix proteins to link the envelope with the virus core would be compensated by the interactions between RNP and glycoproteins since a layer of RNP is situated proximal to the inner leaflet of the membrane which should be the cytosolic tail of the glycoproteins (M Pepin et al., 2010).

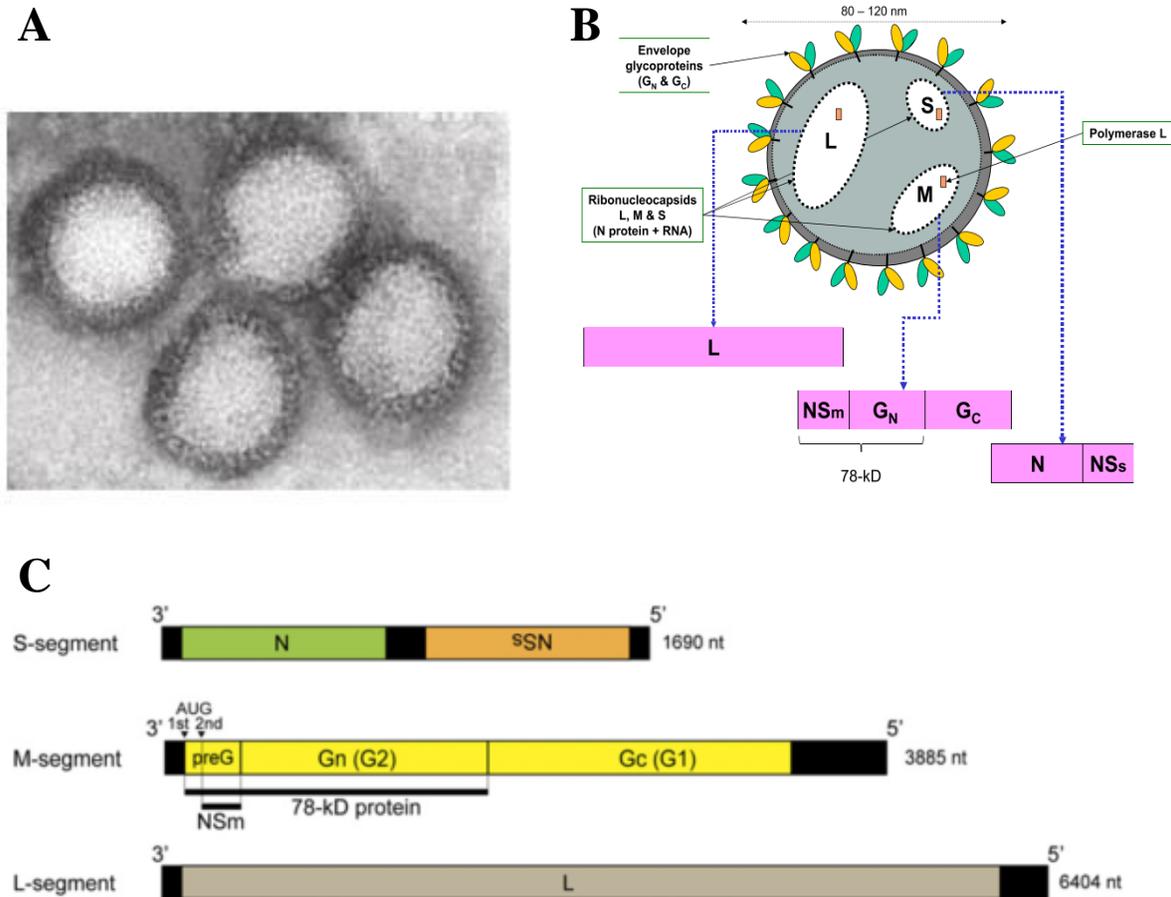


Figure 4 Electron micrograph and Schematic diagram for RVFV virion and genome

A) Electron micrograph of RVFV (40,000 \times). B) Schematic diagram for RVFV virion. C) Schematic diagram for RVFV genome. The S segment encodes the N and NSs proteins in an ambisense manner. The M segment encodes the NSm, the 78-kDa protein and Gn and Gc. The first and second AUG of the M mRNA are in the preglycoprotein region which contains five in-frame AUGs. The 78-kDa and the NSm proteins are synthesized beginning from first and second start codon respectively. The L segment encodes the L protein. Adapted from Pepin, M., Bouloy, M., Bird, B. H., Kemp, A., & Paweska, J. (2010). Rift Valley fever virus (Bunyaviridae: Phlebovirus): an update on pathogenesis, molecular epidemiology, vectors, diagnostics and prevention. *Vet. Res.*, 41(6), 61, and Ikegami, T. (2012). Molecular biology and genetic diversity of Rift Valley fever virus. *Antiviral Res.*, 95(3), 293-310.

1.1.6 Genome

The RVFV genome is composed of three single-stranded RNA segments (Figure 4C): The L segment encodes the viral RNA-dependent RNA polymerase (RdRp) (L) protein; the M segment encodes the 78 kD protein, NSm proteins and protein precursors which are processed into the mature glycoproteins Gn and Gc; the S segment encodes the nucleoprotein (N) and NSs protein in an ambisense manner (CS Schmaljohn & Hooper, 2001). The M mRNA contains five in-frame AUGs (M Pepin et al., 2010). The NSm proteins are synthesized from the first and second AUG of the M mRNA in the preglycoprotein region (Elliott, 2013).

1.1.7 Functions of RVFV proteins

L protein

The L protein (237.7 kDa), which is responsible for viral mRNA and genomic RNA synthesis, is encoded by the L-segment and packaged into the virions (Muller, Poch, Delarue, Bishop, & Bouloy, 1994; Piper, Sorenson, & Gerrard, 2011). As an RNA polymerase related to negative-stranded RNA virus, L protein plays a crucial role in synthesizing primary transcripts and initiating the infectious cycle in infected cells (Ikegami, Won, Peters, & Makino, 2005; Lopez, Muller, Prehaud, & Bouloy, 1995). In the RVFV L protein, identified functional motifs such as Regions 1, 2, and 3 (aa 60–185, aa 650–788, and aa 895–1206) are conserved among different RVFV strains (Bird, Khristova, Rollin, Ksiazek, & Nichol, 2007; Ikegami, 2012).

N protein

The N protein is encoded in antisense by the S segment which has ambisense polarity (Collett et al., 1985; Struthers, Swanepoel, & Shepherd, 1984). The N protein along with the viral genomic RNA and L protein can be packaged into an RNP complex with a flexible serpentine-like structure (Raymond, Piper, Gerrard, & Smith, 2010). RNA binding cleft was

identified on the N protein, but the precise organization of RNA, N and L proteins in the RNP is still unclear (Ferron et al., 2011). All the functions related to the N protein, including viral RNA and antigenome protection, RNA transcription and replication, and virion assembly, involve RNA binding (Ikegami, Peters, & Makino, 2005; Lopez et al., 1995). This fact arouses interest in the identification of antiviral drugs which can inhibit this binding (Ellenbecker, Lanchy, & Lodmell, 2012). The N protein is highly conserved and immunodominant (R Swanepoel, Struthers, Erasmus, Shepherd, McGillivray, Shepherd, et al., 1986). Based on this knowledge, N protein was selected to test antibodies in vaccine studies. Strong humoral and lymphocyte proliferative immune responses were observed in mice immunized with N protein encoding cDNA and 60% of immunized mice survived in a virulent RVFV challenge (Lagerqvist et al., 2009; Wallace et al., 2006a). In addition, a serological diagnostic method using recombinant N protein was developed to detect IgG and IgM antibodies against RVFV by indirect enzyme-linked immunosorbent assays (ELISA) (Jansen van Vuren, Potgieter, Paweska, & van Dijk, 2007).

Glycoproteins Gn and Gc

Glycoproteins Gn and Gc are encoded by the RVFV M segment and are initially translated as protein precursors. The M mRNA contains five in-frame AUGs and the protein precursor generated from the first start codon is cleaved to 78 kD and Gc, while the other precursors generated from the second, third, fourth, or fifth AUG can be cleaved to mature Gn and Gc by the host cellular machinery (Kreher et al., 2014). It is reported that the protein precursor from the second AUG is more efficient than the protein precursor from the third AUG for Gn expression (Suzich & Collett, 1988). The production of mature Gn and Gc production requires two signal peptidase cleavage sites (Gerrard & Nichol, 2007).

The glycoproteins of RVFV have the structural features of *Phlebovirus* including transmembrane domains, cysteine residues, and mature in the Golgi apparatus (Gro, Di Bonito, Fortini, Mochi, & Giorgi, 1997; CS Schmaljohn & Hooper, 2001). Because only Gn has a Golgi localization signal, it is hypothesized that oligomerization of viral glycoproteins is crucial for their accurate transit to Golgi apparatus (S. R. Gerrard & S. T. Nichol, 2002; Shi, Lappin, & Elliott, 2004). However, the Golgi localization signal domain of Gn is only functional in the precursors that start at the second, fourth, or fifth AUG but not in that start from the first start codon (Gerrard & Nichol, 2007).

Glycoproteins Gn and Gc present on the surface of RVF virions and can induce neutralizing antibodies which are critical for protection against virus infection (Besselaar & Blackburn, 1991; Besselaar, Blackburn, & Meenehan, 1991). They are also related to virus attachment (C Schmaljohn & Nichol, 2007). Therefore, glycoproteins of RVFV can be ideal targets for vaccine development (Faburay et al., 2014). It has been reported that a Gn/Gc subunit vaccine can elicit a strong antibody response in sheep (Faburay et al., 2014).

NSs protein

The NSs (31 kDa) protein is translated from the NSs mRNA which is transcribed from the antiviral-sense portion of the S segment (Ikegami, 2012). As a major virulence factor of RVFV, the NSs protein plays a critical role in viral evasion and is responsible for lethal infection in mice (Bouloy et al., 2001; P. Vialat, A. Billecocq, A. Kohl, & M. Bouloy, 2000). NSs proteins are present in the nucleus and cytoplasm of infected cells (Yadani, Kohl, Préhaud, Billecocq, & Bouloy, 1999). To date, there is no report identified the nuclear localization signal in NSs protein which has a filamentous structure in the nucleus (Yadani et al., 1999). The filaments present in the nucleus results in DNA damage responses, cell cycle arrest, p53 activation, and apoptosis. It

has been reported that the ΩXaV motif (ΩXaV motif: Ω , omega: Tryptophan or Phenylalanine, X: any amino acid, a: Aspartic acid or Glutamic acid, V: Valine) is responsible for the filament formation (Cyr et al., 2015) and PXXP motifs (P: proline, X: any amino acid) play a crucial role in NSs protein accumulation in nucleus (Billecocq et al., 2004).

The NSs protein subserves the degradation of two host proteins, the TFIIH p62 and the double-stranded RNA (dsRNA)-dependent protein kinase (PKR) proteins via the cullin 1-Skp1-Fbox E3 ligase complex (Habjan et al., 2009; Ikegami et al., 2009; Kalveram, Lihoradova, & Ikegami, 2011; Le May et al., 2004; Mudhasani et al., 2016). Usually, cellular protein degradation occurs via the ubiquitin (Ub)-proteasome system (Hershko & Ciechanover, 1998; Sorokin, Kim, & Ovchinnikov, 2009). To degrade TFIIH p62 and/or PKR, the protein(s) must be ubiquitinated first (Hershko & Ciechanover, 1998; Komander & Rape, 2012; Randow & Lehner, 2009). Thus, the hypothesis that RVFV NSs protein engages as an adaptor protein in the E3 ligase complex which can promote the transfer of Ub was proposed and is partially proved (Figure 5) (Kainulainen, Lau, Samuel, Hornung, & Weber, 2016; Mudhasani et al., 2016). Also, the NSs protein can directly bind to TFIIH p44 which is one of the ten proteins composed of TFIIH, inhibits the TFIIH assembly which is essential for rRNA synthesis and transcription-coupled DNA repair subsequently (Iben et al., 2002; Le May et al., 2004). On the other hand, PKR is a double-stranded RNA-activated protein kinase induced by interferon and activated by viral dsRNA or ssRNA (Feng, Chong, Kumar, & Williams, 1992; Garcia, Meurs, & Esteban, 2007; Nallagatla et al., 2007). One important function of the activated PKR is to phosphorylate the eukaryotic translation initiation factor 2 (eIF-2), further inhibits cellular and viral mRNA translation (Garcia et al., 2007; Sadler & Williams, 2008). However, the PKR can be stabilized by a proteasomal inhibitor of MG 132 or lactacystin when NSs is presented (Ly & Ikegami,

2016). PKR is mainly found in the cytoplasm, and a few is located in the nucleus. The degradation of this protein in RVFV-infected cells happens in both sites (Garcia et al., 2006; Ikegami et al., 2009).

NSs protein is also responsible for the suppression of interferon (IFN)- β gene regulation (Bouloy et al., 2001; Muller et al., 1995). IFN- β belongs to Type-I IFNs which are essential for innate immunity when the body recognizes a virus invasion. To inhibit IFN- β expression, the NSs protein interacts with Sin3A-associated protein (SAP30) to form a complex of YY1/SAP30/NCoR/Sin3A/HDAC-3 on the IFN- β promoter region (Le May et al., 2008). However, the stability of the NSs gene relies on host alternation (Moutailler et al., 2011). The mutants of NSs protein can cause its failure in suppressing IFN- β gene upregulation (Head, Kalveram, & Ikegami, 2012).

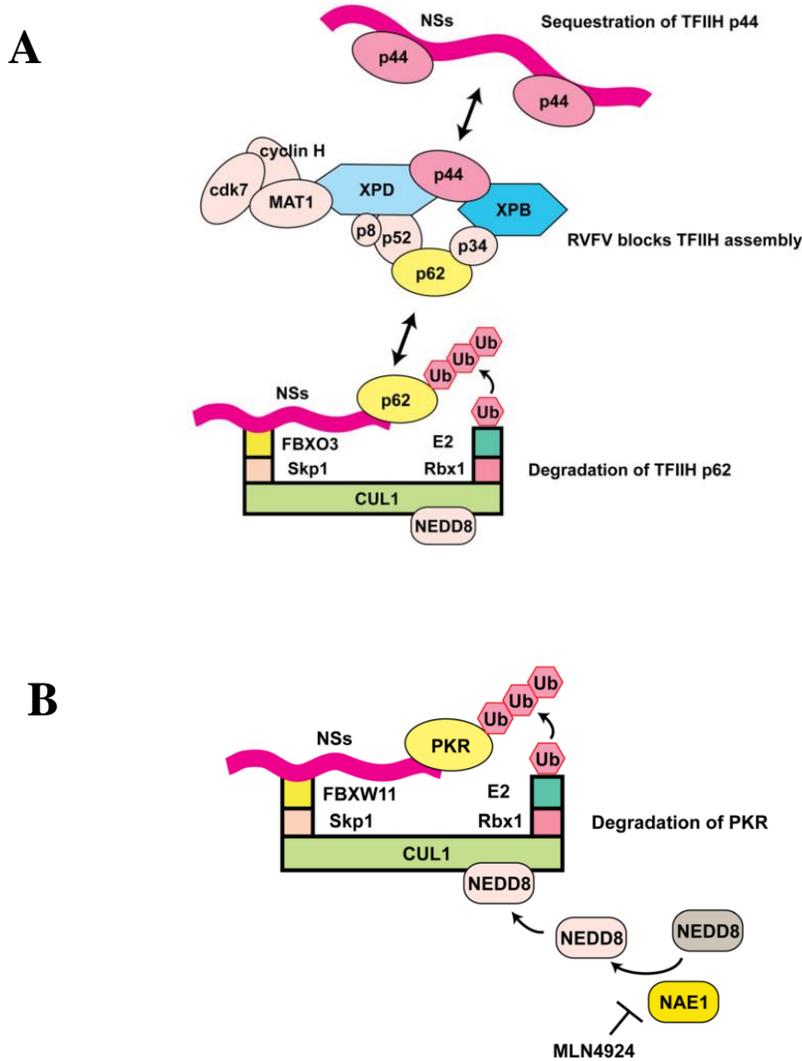


Figure 5. Schematics of RVFV NSs-mediated TFIH suppression and PKR degradation

A) Schematics of RVFV NSs-mediated TFIH suppression. As the picture illustrated, the NSs protein can suppress TFIH by two ways: 1. binds to p44 and impounds it from the assembly site of TFIH subsequently; 2. Formats the E3 ligase complex and promotes the degradation of p62 through RVFV NSs. B) Schematics of RVFV NSs-mediated PKR degradation. RVFV NSs protein forms the E3 ligase complex and further promotes the degradation of PKR via the ubiquitin-proteasome pathway. Adapted from Ly, H. J., & Ikegami, T. (2016). Rift Valley fever virus NSs protein functions and the similarity to other bunyavirus NSs proteins. *Virology Journal*, 13(1), 1-13.

The 78-kD and NSm proteins

The 78-kDa and the NSm (14 kDa) proteins are encoded by the M segment from the first and second AUGs, respectively (Kakach, Suzich, & Collett, 1989; Kakach, Wasmoen, & Collett, 1988; C. Schmaljohn et al., 1989; Struthers et al., 1984). The biological functions of these two proteins are poorly understood. Until 2006, Won S. et al. first demonstrated that the 78-kDa and the NSm proteins are not essential for viral replication in cell culture by using a reverse genetics system to generate mutant viruses lacking the expression of one or both proteins (Won, Ikegami, Peters, & Makino, 2006); this group also reported that the NSm protein has an antiapoptotic function (Won, Ikegami, Peters, & Makino, 2007). Also, a recombinant RVFV with full NSm region deletion is highly attenuated in rats and shows a reduced ability to infect mosquitoes (Crabtree et al., 2012; Kading et al., 2014). While Weingartl H.M. et al. demonstrated that the 78-kDa protein is a structural protein in C6/36 mosquito cell generated virus but not in Vero E6 derived virus (Weingartl, Zhang, et al., 2014). They hypothesized that the 78-kDa protein might play a major role in transmission from the mosquitoes to the ruminant host (Weingartl, Zhang, et al., 2014).

1.1.8 Animal models for RVFV

Animal models are important to study the pathogenesis of RVFV and evaluate therapeutic and prophylactic interventions against the virus infection. Various animal species, including the virus targeted ruminants, rodents, and non-human primates, were used to conduct RVFV research.

1.1.8.1 Ruminants

Ruminants can be reliable animal models since they are naturally very susceptible to RVFV infection. The evaluation of pathology, virology, and immunology for the infection can

easily be performed. The severity of the disease induced by virulent RVFVs is not only varied in young animals and adults but also depending on the virulence of the RVFV strain (Raymond et al., 2010). Hepatic lesions, such as swollen, mottled appearance, and necrosis can be observed in neonatal lambs after RVFV infection (G. Anderson, Jr., Lee, et al., 1991). Disease in RVFV infected adult sheep usually mild and subacute (C Schmaljohn & Nichol, 2007). Early-onset viremia with a transient pyrexia and mild hepatic lesions were observed in 4-5 month old sheep which were inoculated with RVFV subcutaneously (Faburay, Gaudreault, et al., 2016). Consistent results were obtained from 4-5 months old cattle RVFV challenge model (W. Wilson et al., 2016). Because the limitation of the space in BSL-3 facilities and only limited numbers of qualified experimental ruminants can be obtained in one study, less virulent RVFV studies have been performed on ruminants than rodents.

1.1.8.2 Rodents

Rodents are used to study the pathogenesis of RVFV and to evaluate vaccines and antiviral drugs against this zoonotic pathogen. Different rodent species, such as mice, rats, hamsters, and gerbils have been used as animal models for RVFV infection (G. Anderson, Slayter, Hall, & Peters, 1990; G. Anderson, T. Slone, & C. Peters, 1987; G. Anderson, Slone, & Peters, 1988; Kende, Alving, Rill, Swartz, & Canonico, 1985; Morrill et al., 1987). Because of the absence of important clinical signs, such as on-set hepatitis and delayed encephalitis caused by RVFV infection, showed in hamster and gerbil models, they have not been extensively used as models for RVFV studies (G. Anderson et al., 1990; G. Anderson et al., 1988).

Mouse models

In mouse models, virulent RVFV infection leads to acute hepatitis and encephalitis (Bhardwaj, Heise, & Ross, 2010; Flick & Bouloy, 2005; Ikegami & Makino, 2011). As the

overall disease developed by the virulent RVFV infection in mice is quite similar with RVFV pathology in humans, mice are excellent small animal models to study pathogenesis, vaccine efficacy, and antiviral drugs for RVFV (Boshra, Lorenzo, Busquets, & Brun, 2011; Ikegami & Makino, 2009, 2011). After challenge with a virulent RVFV via peripheral routes, BALB/c mice manifest clinical signs such as ruffled fur, decreased body weight, lethargy, and death. The clinical symptoms could be observed rapidly, usually less than 7 days. While some mice can succumb within three days post infection (dpi) without the development of clinical signs (Mandell et al., 2010). Acute viral hepatitis can be observed in both mice and humans since the primary site of RVFV-induced lesions is in the liver. Because of the apoptosis induced by RVFV infection, a lot of hepatocytes can be detected by Hematoxylin and eosin (H&E) as early as 3 dpi (D. Smith et al., 2010) stain. Meanwhile, the liver enzymes and bilirubin in the blood increase due to the infection as well (D. Smith et al., 2010).

Although liver seems to be the early dominant target of RVFV, during severe infections, the virus can be detected in most tissues and cell types (Näslund et al., 2008; D. Smith et al., 2010). In both mice and human severe fatal cases, development of delayed encephalitis was observed after acute hepatitis caused by RVFV (Van Yelden, Meyer, Olivier, Gear, & McIntosh, 1977).

Different infection routes of exposure in mouse model can also induce different severity of the disease. Compare with the peripheral routes, BALB/c mice with virulent RVFV aerosol exposure develop more neurologic clinical signs, such as cage circling or hind limb paralysis (Reed et al., 2013). But acute hepatitis is observed despite the routes of exposure. Ribavirin, an effective antiviral drug which can provide 80-90% protection from lethal challenge via the peripheral route, can hardly protect mice with lethal RVFV aerosol exposure (Morrill et al.,

2010; Reed et al., 2013). These results indicate that early RVFV invasion of brain tissues via aerosol exposure likely account for the failure of ribavirin to protect mice from mortality.

Meanwhile, aerosol exposure of BALB/c mice with RVFV virulent strains shrinks the mean time to death compared with the peripheral exposure, from 3 dpi to 5 dpi or longer (Ross, Bhardwaj, Bissel, Hartman, & Smith, 2012).

Immunodeficient mouse models contribute the research on RVFV cell-mediated immune responses which aspect has limited literature. *In vitro* studies demonstrated that virus replication was not affected no matter IFN- γ was present or not (Habjan et al., 2009; Nfon, Marszal, Zhang, & Weingartl, 2012). The *in vitro* results were corresponding to the *in vivo* study which showed that all IFN γ R/- mice were survived after MP12 challenge while all IFNAR $^{-/-}$ mice were dead due to infection (Bouloy et al., 2001). Among these innate immune factors, it is not surprising that type I IFN that plays a critical role in RVFV infection (Weber, Elliott, Brasier, Garcia-Sastre, & Lemon, 2009). Because type I IFN is synthesized by the virus-infected cells to inhibit viral replication and stimulates the neighboring cells to express antiviral factors, subsequently preventing the spread of the viral infection (Samuel, 2001). Moreover, the IFN-induced antiviral factors can trigger apoptosis of the infected cells and they are essential modulators of adaptive immunity (Wilkins & Gale, 2010). Different *in vitro* and *in vivo* studies indicate that type I IFN plays a crucial role in RVFV infection. When type I IFN is present/absent in cell culture, RVFV replication was impaired/enhanced (G. Anderson & Peters, 1988; Do Valle et al., 2010; Ermler et al., 2013). While better outcomes were showed in the animals that were treated with IFN or IFN inducers prior/after the RVFV infection (Morrill, Jennings, Cosgriff, Gibbs, & Peters, 1989; Peters, Reynolds, Slone, Jones, & Stephen, 1986). Thus, during the first crucial stage of RVFV infection, type I IFN response is indispensable to limit the viral spread (Weber & Elliott, 2002).

While reduced viremia was observed in monkeys which were given IFN- γ prior to infection compared to non-treated animals (Morrill, Czarniecki, & Peters, 1991). IFN- γ is predominantly synthesized by natural killer (NK), natural killer T (NKT) cells, Th1 cells, and CTL effector T cells. So IFN- γ is crucial in both innate immunity and adaptive immunity (Goodbourn, Didcock, & Randall, 2000). As high IFN- γ response was observed after restimulation of cells from convalescent goats and sheep, and cell-mediated immunity played a role in post-vaccinal protection against RVFV infection in mice, cell-mediated immunity could be involved in the long-term protection against RVFV (Boshra, Lorenzo, Rodriguez, & Brun, 2011; Busquets et al., 2010; Nfon et al., 2012).

Rat models

Rats are also susceptible to RVFV infection and have been used in research since the early 1930s (Findlay & Daubney, 1931). In the natural environment, mortality of rats occurs during outbreaks of RVF, although there is no evidence that showed these rats had a direct infection (Daubney & Hudson, 1933; Weinbren & Mason, 1957); indicating that rats could serve as an intermediate host for the RVFV during outbreaks.

Susceptibilities to infection differ among different rat strains when infected with the same virulent RVFV strain (G. Anderson, Jr., Rosebrock, Johnson, Jennings, & Peters, 1991; Peters & Anderson Jr, 1981; Peters & Slone, 1982). Despite challenging routes, Wistar-Furth and Brown Norway rats are exquisitely susceptible to ZH501 with a median lethal dose of 1-5 plaque forming unit (PFU) (Peters & Anderson Jr, 1981; Peters & Slone, 1982). These two rat strains died within 3–5 days with fatal lesion of hepatic necrosis. The virus is found in many organs, such as lung, liver, spleen, and kidney (G. W. Anderson, Jr., T. W. Slone, Jr., & C. J. Peters, 1987; Peters & Anderson Jr, 1981). High levels of viremia are detected as well (Bird et al.,

2008). ACI and MAXX rats are moderately susceptible to ZH501 when challenged with 5×10^3 to 5×10^5 PFU and died within 2 to 3 week with fatal encephalitis. The mortality rate is 50% after peripheral infection (Bucci, Moussa, & Wood, 1981; Peters & Slone, 1982). At the late stage of disease, virus and lesions can be only detected in the brains of rats with neurologic symptoms including circling in the cage, hind limb paralysis, head tremors, head tilt, and ataxis (Bucci et al., 1981; Peters & Slone, 1982). Although no clinical signs are observed in Lewis strain after ZH501 exposure, viremia and immune responses specific to RVFV infection are detected (G. Anderson & Smith, 1987; Peters & Anderson Jr, 1981; Peters & Slone, 1982). Additionally, virus replication occurs in the liver and spleen. While Buffalo, DA, and Fischer rats resist to ZH501 following infection subcutaneously with 5×10^5 PFU (Peters & Slone, 1982). Inbred rat strains can display more differences in lethality reproducibly compared with inbred mouse strains (G. Anderson, Jr., Rosebrock, et al., 1991; Peters & Anderson Jr, 1981; Peters & Slone, 1982). Different strains of RVFV show million fold difference in virulent when to challenge the Wistar-Furth rats (G. Anderson & Peters, 1988).

1.1.8.3 Non-human primates

Since RVF is a zoonotic disease, non-human primate models of RVF is crucial in investigating antiviral drugs and vaccines for human use. However, non-human primate models with RVFV infection does not produce a repeatable fatality. Most of the primates present mild clinical signs, such as a decrease in activity, viremia, fever, leukocytosis, followed by leukopenia (Findlay & Daubney, 1931; Ross et al., 2012). Rhesus macaques are the first used non-human primate model since 1931 and considered to be the best model when infecting with ZH501 to demonstrate the range of symptoms observed in humans (Findlay & Daubney, 1931; Peters & Linthicum, 1994; Ross et al., 2012). Infected rhesus macaques showed liver lesion characterized

by mid-zonal pattern, which is similar in human cases (Van Yelden et al., 1977). It has been shown that IFN response is critical to prevent a fatal outcome in RVFV infected rhesus macaques and IFN- α is effective to prevent disease (Morrill, Czarniecki, et al., 1991; Morrill et al., 1990). Different exposure routes were researched and the results demonstrated that infection with aerosol exposure is slightly more susceptible than infection with peripheral exposure (B. Easterday, 1965). Neurotropic RVFV infection to rhesus macaques showed no febrile reaction after IP inoculation; however, fatal infection was observed in intracerebrally or intranasally inoculated rhesus macaques (Findlay, MacKenzie, & Stern, 1936).

Lethal non-primate infection models are also needed to demonstrate the effectiveness of newly developed antiviral drugs and vaccines. In addition, RVFV rhesus macaque model needs intravascular (IV) infection which is different from the natural exposure route of extravascular infection transmitted by mosquitoes (Turell & Spielman, 1992). Recently, common marmoset (*Callithrix jacchus*) was used to evaluate the infectivity and pathogenicity of ZH501 by IV, subcutaneous (SC), and intranasal exposure routes (D. Smith et al., 2012). Results demonstrated that the overall disease of infected Marmoset was more severe than rhesus macaques. When infected virus via IV and SC routes, the major consequence of RVFV infection is an overwhelming infection of hepatocytes. Neurological impairment and hemorrhagic manifestations were observed as well (D. Smith et al., 2012). Both African green monkeys and Marmosets are susceptible to RVFV via aerosol exposure and developed fatal encephalitis (Hartman et al., 2014). H&E staining demonstrated meningoencephalitis caused by RVFV infection in both species (Hartman et al., 2014). It is worth to note that all these non-human primate studies used small sample sizes and no in-depth questions have been answered. These models should be tested further in validating candidate antiviral drugs or vaccines.

Other species of non-human primates, such as South American, Indian monkeys, spider monkeys, and baboons were evaluated for susceptibility of RVFV by different groups (B. Easterday, 1965; Findlay, 1932). Recombinant RVFV vaccines using vaccinia virus as a vector to express Gn and Gc of RVFV were tested in the baboon model and protective levels of antibody were induced (Papin et al., 2011).

1.1.9 RVFV vaccines

As a strategy for prophylaxis, vaccination plays a critical role to impede the invasion of microbiologic pathogens. Niklasson et al. demonstrated that the humoral immunity induced by vaccines is sufficient for protection against RVFV (BS Niklasson, Meadors, & Peters, 1984). In addition, the RVFV strains are relatively conserved at the nucleotide and amino acid levels (Bird, Khristova, et al., 2007). Unfortunately, there is currently no fully licensed RVFV vaccine available in the U.S. for human or animal use. Currently, several groups are focusing on the RVFV vaccine development using different strategies.

The fundamental mechanism for vaccination is based on the adaptive acquired immune system. It can remember the specific pathogens that have been encountered in the past for a period and once that pathogens invade the organism again, it reacts faster and more fiercely (Abbas, Lichtman, & Pillai, 2012). The adaptive immune system is subdivided into cell-mediated immunity and humoral immunity based on the factors and cells involved. Cell-mediated immunity is mediated by antigen-specific cytotoxic T-lymphocytes and requires the activation of phagocytes and the release of various cytokines. The main place of cell-mediated immunity is inside cells. While the humoral immunity is mediated by macromolecules that exist in extracellular fluids including secreted antibodies, complement proteins, and antimicrobial peptides. The humoral immunity fights against pathogens outside the cells (Abbas et al., 2012).

The specific adaptive immune response is triggered and initiated by antigen presenting cells (APCs) including dendritic cells and macrophages. APCs can engulf and digest viruses, transport the viral antigens to regional lymph nodes, and present them on major histocompatibility complex (MHC) molecules to naive T cells. These naive T cells will differentiate into effector cells once the relevant factors are present and the T-cell receptor is specific for the antigen. Because of the structure difference, the MHC class I molecules can be recognized by cytotoxic T lymphocytes (CD8+) which can differentiate into cytotoxic T lymphocytes (CTLs); while MHC class II molecules can be recognized by Helper T lymphocytes (CD4+) which can differentiate into different T helper (Th) cells. There are two major subtypes of Th cells, Th1, Th2 cells. Th1 cells are necessary for clearing intracellular pathogens, while Th2 cells are the host immunity effectors against extracellular organisms. Th cells can also produce distinct sets of cytokines which are important in cell signaling (Chaplin, 2010).

The primary functions of CTLs are to identify and kill the virus-infected cells (Abbas et al., 2012). Because a wide range of cells can express MHC class I molecules which can recognize by CTLs, most of the virus-infected cells can report their situations to CTLs by presenting MHC I/peptide complexes on their cell surface. Upon recognition of infection, the CTL secretes cytotoxins perforin, granzymes, and granulysin. Granzymes enter the cytoplasm after the perforin created pores in the membrane and their serine protease function activates the caspase cascade that subsequently leads to apoptosis (Abbas et al., 2012).

B cells play a crucial role in humoral immunity and they can recognize different antigens by their Ig receptor on the membrane. Once the receptor-recognition of an antigen happens, it activates B cells and the cell begins to proliferate and produce IgM. This process takes several days for RVFV infection. Then the activated B cell processes the antigen to peptides and further

presents the peptides by displaying them on MHC class II molecules to activated Th cells. After that, the interacting Th cell secretes different signals to the B cell in order to make it proliferate. In this process, the secretion of antibodies shifts from IgM or IgD isotypes to IgA, IgE, or IgG (Abbas et al., 2012). Although part of the B cells differentiates into memory B cells which do not secrete antibodies to combat the invading pathogen, they are ready to give a faster response if the specific antigen appears again. If the organism is exposed to a specific antigen prolonged or repeatedly, antibodies with higher affinity for that particular antigen will be produced. As the secreted antibodies enter the blood, they can perform their functions throughout the organism. Antibodies are quite useful to combat pathogens in three main ways: 1). coat pathogens and target them for phagocytosis; 2). stimulate other parts of the immune system in order to destroy the pathogens; 3). bind to the pathogens directly to inhibit the infection.

Antibodies are essential in clearance of the RVFV during the infection and in the prevention of re-infection in convalescent or immunized animals (Busquets et al., 2010; M Pepin et al., 2010). RVFV was cleared from the blood gradually after the IgM and IgG antibodies appear in experimentally-infected animals. Among the IgGs, neutralizing antibodies which have high affinities to some specific structures on the surface of the RVFV can inhibit the interaction between the virus and receptor binding. Thus, the cells are protected from infection (Reading & Dimmock, 2007). Although viremia was not able to be observed before the detection of neutralizing antibodies in experimentally-infected sheep (Busquets et al., 2010), the close times for the appearance of neutralizing antibodies and viral clearance indicates that antibodies along with the innate immunity are necessary for controlling RVFV infection (G. Anderson et al., 1987; Morrill et al., 1990). As mentioned before, glycoproteins Gn and Gc can elicit protective neutralizing antibodies. Non-immunized animals can be protected from infection after

administration of neutralizing monoclonal antibodies and new-born of immunized animals are protected via suckling (G. Anderson et al., 1987; Bhardwaj et al., 2010; Peters et al., 1986; Smithburn, 1949).

The formalin-inactivated RVFV vaccines have a long history since 1962 (Randall, Gibbs, Aulisio, Binn, & Harrison, 1962). For veterinary use, one formalin-inactivated RVFV vaccine derived from a pantropic RVFV strain can significantly reduce the rates of abortion and mortality but still cannot entirely protect the whole population (Barnard & Botha, 1977; ITI Gemnoranda, 1983). Because RVF is a zoonosis, humans can be threatened during an outbreak. Thus, the NDBR103, one of the earliest inactivated vaccines was developed for human use. NDBR103 can induce high titers of neutralizing antibodies in most of a group of UN soldiers after three-dose vaccination (Randall et al., 1962). Based on this knowledge, TSI-GSD 200 was further developed and is used for at-risk laboratory workers in the U.S. provided by the U.S. Army Medical Research Institute of Infectious Diseases (USAM-RIID) recently (Pittman et al., 2016). However, although inactivated vaccines are highly safe, boosters are required to get a long-term immunity which means limited access to this vaccine (Pittman et al., 1999; Rusnak, Gibbs, Boudreau, Clizbe, & Pittman, 2011). These two formalin-inactivated RVFV vaccines can induce immunization side effects such as erythema, swelling, tenderness or pain at the injection site (Kark, Aynor, & Peters, 1982; B Niklasson, 1982). Due to these reasons, live attenuated vaccines were developed for demand in animal and human use.

The first live attenuated vaccine, Smithburn strain, was isolated from a mosquito and serially passaged in mouse brains/eggs (Smithburn, 1949). Although this vaccine is significantly attenuated, 29% of vaccinated pregnant cows showed abortion (Botros et al., 2006). This fact raised the concern that Smithburn strain was not fully attenuated. In addition, as a live vaccine,

the Smithburn strain has a potential for reversion (Sall et al., 1999). Thus, the Smithburn strain is not recommended for use in RVFV non-endemic countries (Ikegami & Makino, 2009).

Currently, two RVFV live attenuated vaccines, MP12 and Clone 13, are in clinical trials. The temperature-sensitive MP12 strain (Saluzzo & Smith, 1990) was obtained through 12 serial passages of a virulent isolate ZH548 in the presence of 5-fluorouracil and has been shown to be safe and efficacious against virulent RVFV challenge in sheep (Morrill et al., 1987), cattle (Morrill, Mebus, & Peters, 1997) and macaques (Morrill & Peters, 2003). Immunization of pregnant ewes with MP12 induced protective neutralizing antibodies without fetal abnormality (Baskerville, Hubbard, & Stephenson, 1992; Morrill, Carpenter, et al., 1991; Morrill, Johnson, et al., 1991). However, one group claimed that this vaccine still can cause abortion in ewes when vaccinated on the 28th day of pregnancy (Hunter, Erasmus, & Vorster, 2002). MP12 can also elicit high neutralizing antibody titers in alpacas but still pathogenic since mild multifocal necrotizing hepatitis was observed at 3 dpi (Rissmann et al., 2017). Currently, MP12 is the only conditionally licensed vaccine for use for veterinary purposes in the U.S (Ikegami et al., 2015) and just went through the Phase 2 clinical trial in humans (Pittman et al., 2016). After a single dose of vaccination, 8 of 9 vaccinees (89%) maintained an 80% plaque reduction neutralization titer (PRNT₈₀) \geq 1:20 after five years and no reversions of amino acids were observed by RNA sequencing (Pittman et al., 2016). Ikegami et al. demonstrated that the MP12 attenuation is caused by a combination of multiple partial attenuation mutations on different segments, suggesting that a single reversion mutation is hard to result in a reversion to virulence (Ikegami et al., 2015).

On the other hand, the Clone 13 is a plaque isolate with approximately 70% of the NSs ORF deletion and it is naturally significantly attenuated (Muller et al., 1995). No apparent side

effects were observed in experimental vaccine trials in both mice and ruminants, suggesting that the Clone 13 is safe to use (Dungu et al., 2010; P Vialat, A Billecocq, A Kohl, & M Bouloy, 2000; Von Teichman et al., 2011). Although one study in pregnant sheep induced a protective immunity without abortions (Dungu et al., 2010), Makoschey B. et al. demonstrate that the Clone 13 can cross the ovine placental barrier, resulting in malformations and stillbirths, suggesting Clone 13 could be applied to lambs but not to pregnant animals (Makoschey et al., 2016).

As the reverse genetic system was established for RVFV in 2005, various recombinant RVFVs were developed as vaccine candidates and further evaluated in rodents and ruminants (Bird et al., 2008; Bird et al., 2011; Ikegami, Won, Peters, & Makino, 2006; Morrill et al., 2013; Weingartl, Nfon, et al., 2014). To enhance the safety of the MP12 vaccine, a single-cycle replicable MP12 was generated (Murakami, Terasaki, & Makino, 2016). This recombinant RVFV carries a normal L RNA of MP12, an S RNA encoding green fluorescent protein to replace the NSs protein, and an M RNA encoding a mutant envelope protein lacking an endoplasmic reticulum retrieval signal and defective for membrane fusion function (Murakami et al., 2016). This platform of the reverse genetic system can also benefit the development of DIVA vaccines and studies in molecular virology of RVFV (Ikegami et al., 2006).

The drawback of live attenuated vaccines is the potential for reverse. As RVFV is a segmented RNA virus, reassortment could happen (J. Liu et al., 2017). Therefore, a live attenuated vaccine strain may reassort with a wild strain, resulting in an increase of virulence. Aside from the RVFV itself, other viral vectors which can express foreign antigens also could be used to generate recombinant RVFV vaccines. The glycoproteins of RVFV are considered to be immunogenic and can elicit robust neutralizing antibodies in animals (Besselaar & Blackburn, 1991; Dalrymple, Hasty, Kakach, & Collett, 1989; Keegan & Collett, 1986). Therefore,

Venezuelan equine encephalitis virus (VEEV), lumpy skin disease virus (LSDV), vaccinia, and a replication-deficient chimpanzee adenovirus were used as vectors to express the glycoprotein(s) and protective antibodies were induced in animals (Boshra et al., 2013; Busquets et al., 2014; Gorchakov et al., 2007; Papin et al., 2011; Wallace et al., 2006b; Warimwe et al., 2013). These studies demonstrated that viral vectors could be useful tools to deliver RVFV glycoproteins to the host and have potentials to be veterinary vaccines in the future.

Other strategies to develop RVFV vaccines, including subunit vaccines, DNA vaccines, and transgenic plants have been developed (Bhardwaj et al., 2010; Faburay et al., 2014; Kalbina et al., 2016; Lagerqvist et al., 2009; Näslund et al., 2009; C. S. Schmaljohn et al., 1989; Spik et al., 2006). These vaccines are DIVA compatible. But most of them still need to be improved in immunogenicity and inoculation times. The glycoprotein subunit vaccine which elicited high neutralizing antibody titers without viremia and fever can protect sheep from lethal RVFV challenge (Faburay, Wilson, et al., 2016). However, this vaccine still needs a booster and the efficacy of it in other susceptible animals and non-human primates is still unknown.

1.1.10 Diagnostic methods for RVFV

RVFV can be detected by classical virological methods including histopathology, virus isolation (G. Anderson et al., 1989), detection of antigen and antibodies (Fukushi et al., 2012; Kortekaas et al., 2013; J Meegan et al., 1989; R Swanepoel, Struthers, Erasmus, Shepherd, McGillivray, Erasmus, et al., 1986; van der Wal et al., 2012), and molecular assays (Bird, Bawiec, Ksiazek, Shoemaker, & Nichol, 2007; Le Roux et al., 2009; Sall et al., 2002; W. Wilson et al., 2013).

Hepatic lesions characteristic of RVFV can be observed in the infected host by histopathology test since the liver is the target organ of RVFV for both humans and animals. The

RVFV infection can be further confirmed by immunostaining using specific antibodies (G. Anderson et al., 1989; Coetzer, 1982). However, technical expertise is needed for this approach. RVFV can be isolated from whole blood/serum collected during the acute (febrile) stage of the disease and from the post-mortem samples (G. Anderson et al., 1989). But BSL-3 or higher level laboratories are required to perform this approach (Bird et al., 2009). Currently, a combination of serological and molecular methods is preferred in laboratory diagnosis.

The gold standard serological assay for RVFV is the virus neutralization assay (VNA). Unfortunately, this approach can only be performed in BSL-3 facilities with the live virulent virus for the safety concern (Mansfield et al., 2015). Enzyme-linked immunosorbent assay (ELISA) is commonly used to confirm RVFV infection. Several assays are available for the detection of both RVFV antigens and anti-RVFV antibodies (Fukushi et al., 2012; BS Niklasson & Gargan 2nd, 1985; Paweska, Mortimer, Leman, & Swanepoel, 2005; Williams et al., 2011). Although cross-reaction between RVFV and other *phleboviruses* may happen, both the VNA and ELISAs are considered to be highly specific (M Pepin et al., 2010).

Molecular methods are economical and rapid to detect the viral RNA. Therefore, a number of highly sensitive nucleic acid based molecular tests have been developed and proven useful during RVF outbreaks (Gerdes, 2004). However, expensive equipment and special techniques are needed which hindered the use of molecular methods in the field. Therefore, further studies to develop a diagnostic method for RVFV should focus on simplification of procedure and application on site of outbreaks.

1.1.11 Antiviral drugs for RVFV

Currently, there are no licensed antiviral drugs approved for RVFV treatment in both humans and animals.

Ribavirin, a nucleoside analogue, can inhibit RVFV replication (Oestereich et al., 2014). However, ribavirin can hardly penetrate the blood-brain barrier, resulting in the failure to prevent delayed-onset neurologic disease. Moreover, a shift in disease characteristics from sudden-onset hepatic disease to delayed-onset neurologic disease was observed in animals which were infected with RVFV and further treated with ribavirin (Peters & Anderson Jr, 1981). Thus, ribavirin should only be considered in the treatment of complex RVF.

Favipiravir (T-705) is a broad spectrum inhibitor against a number of different RNA viruses, such as influenza A virus, RVFV, Ebola virus, Paramyxoviruses, Human Metapneumovirus, Arenaviruses, and other Bunyaviruses, etc. in animal models (Caroline et al., 2014; Furuta et al., 2013; Jochmans et al., 2016; Oestereich et al., 2014; Scharton et al., 2014). As a novel viral RNA polymerase inhibitor, Favipiravir-RTP, the active format of Favipiravir in cells, is significantly more selective for the influenza virus RNA-dependent RNA polymerase than human DNA-dependent RNA polymerase (Furuta et al., 2013). Administration of Favipiravir showed promising results in inhibiting replication of RNA viruses in animals; clinical trials should be scheduled soon.

In conclusion, development of antiviral compounds with improved safety and efficacy is in urgent need for treatment of RVF.

Chapter 2 - Mouse Model for the Rift Valley Fever Virus MP12

Strain Infection

The data in this section has been published in *Veterinary Microbiology* (2016) 195: 70-77 by Yuekun Lang, Jamie Henningson, et al., Mouse model for the Rift Valley fever virus MP12 strain infection.

2.1 Introduction

Rift Valley fever virus (RVFV), the causative agent of Rift Valley fever (RVF), belongs to the genus *Phlebovirus*, in the *phenuiviridae* family and is classified as a Category A pathogen designed by the NIH/NIAID (D. Bishop & Shope, 1979; Georgiev, 2009). RVF is an acute, fever-causing zoonotic disease that affects humans and animals such as cattle, buffalo, sheep, goats, and camels. RVFV was first reported in 1931 from a farm of Rift Valley in Kenya (Daubney et al., 1931). The virus has been circulating in the countries of the African continent and Madagascar, causing great economic loss and human deaths. The first RVF outbreak outside Africa was confirmed in 2000 in the Arabian Peninsula, resulting in loss of livestock and more than 200 human deaths out of approximately 2,000 hospitalized patients (Balkhy & Memish, 2003; Shoemaker et al., 2002). This fact raised concern that the disease could spread worldwide (Ahmed et al., 2009).

The RVFV MP12 attenuated strain provides a BSL-2 model virus for preliminary investigations of RVFV prior to using virulent RVFV strains that are required to be handled in an expensive high biosecurity BSL-3 facility (Gaudreault et al., 2015). This attenuated strain was obtained through 12 serial passages of a virulent isolate ZH548 in the presence of 5-fluorouracil and has been shown to be safe and efficacious against virulent RVFV challenge in sheep (Morrill, Carpenter, et al., 1991; Morrill et al., 1987), cattle (Morrill et al., 1997) and macaques

(Morrill & Peters, 2003). However, a previous study showed that this vaccine still can cause abortion in pregnant ewes (Hunter et al., 2002). MP12 is classified as Risk group 2 pathogen and commonly used in BSL-2 laboratories (Ikegami et al., 2015). MP12 is highly attenuated in mice at immunogenic doses (Caplen, Peters, & Bishop, 1985). However, it may cause tissue pathology in some strains of mice such as BALB/c and C57BL/6 mice when administered at high doses (Indran et al., 2013; Lihoradova et al., 2013). When RVFV natural hosts, such as cattle and lambs, were infected with a higher dose (10^6 PFU) of MP12 virus, clinical disease and viremia were not observed in inoculated animals (W. C. Wilson et al., 2014), indicating that they are not good models for evaluating antivirals using RVFV MP12 virus in a BSL-2 environment. It has been reported that all strains of RVFV remain closely related at the nucleotide and amino acid level (Ikegami, 2012), suggesting that antiviral drugs or vaccines effective against one strain may also be effective against all strains. To date, there is no fully licensed vaccine for human or animal use in the U.S., and effective antiviral drugs have not been identified (Eddy, Peters, Meadors, & Cole Jr, 1981; Hunter et al., 2002; Ikegami, 2012; L. Liu, Celma, & Roy, 2008). It is urgent to develop efficacious antivirals and vaccines to protect public and animal health. However, limited laboratories can perform efficacy testing of RVF antivirals and vaccines and have access to the virulent RVFV strains, since high biocontainment facilities are required and the cost for performing the antiviral test is also very expensive. Based on above knowledge, our objective was to establish a mouse model susceptible to infection with the MP12 vaccine strain in order to identify and evaluate antiviral compounds against RVFV in a BSL-2 level facility. BALB/c mice are highly susceptible to the virulent RVFVs such as the ZH501 strain, displaying acute-onset liver disease and delayed-onset encephalitis (D. Smith et al., 2010). Mouse strains including NZW/LacJ, 129S1/SvImJ, and C57BL/6J have been reported to be susceptible to

infection with Punta Toro virus, another phlebovirus in the *Phenuiviridae* family (Ashley et al., 2011; Mendenhall, Wong, Skirpstunas, Morrey, & Gowen, 2009). Furthermore, MP12 is able to infect and cause mortality in Sv129 IFN α/β receptor $-/-$ (interferon α/β receptor knockout) mice that have been used to study vaccine efficacy (Boshra, Lorenzo, Rodriguez, et al., 2011; Bouloy et al., 2001; Lorenzo, Martin-Folgar, Hevia, Boshra, & Brun, 2010). Therefore, we selected and screened six strains of mice [129S6/SvEv, STAT-1 KO (129S6/SvEv-Stat1^{tm1Rds}), 129S1/SvImJ, C57BL/6J, NZW/LacJ, and BALB/c] to test their susceptibility to the MP12 strain in this study. The mouse strain most susceptible to the MP12 vaccine strain infection will likely be selected for further testing the efficacy of antiviral compounds in future studies.

2.2 Materials and Methods

Virus and Cells

African green monkey kidney epithelial (Vero) E6 cells (ATCC-CRL 1586) were grown in 1x DMEM medium containing 10% fetal bovine serum (FBS, Atlanta Biologicals). The RVFV MP12 strain was kindly provided by the US Army Medical Research Institute for Infectious Diseases. The virus was propagated and passaged three times in Vero E6 cells. Cells were infected with 0.01 multiplicity of infection (MOI) of the virus in the medium of DMEM with 10% FBS. Cell cultures were collected when 80–95% of the infected cells showed cytopathogenic effect (CPE). The titer of viral stock was determined and calculated as tissue culture infective dose 50 (TCID₅₀) based on the presence of cytopathic effect.

Animals

Six groups (14 mice/group) of seven-week-old female mice were obtained from different companies. 129S6/SvEv and STAT-1 KO mice were purchased from Taconic, Hudson, NY; 129S1/SvImJ, C57BL/6J and NZW/LacJ mice were purchased from Jackson Lab, Harbor, ME;

BALB/c mice were purchased from Charles River Laboratories, Wilmington, MA. All animal procedures used in this study complied with guidelines set by the USDA and were approved by Kansas State University Animal Care and Use Committee.

Mouse Infection Experiment

Mice were maintained in a mouse vivarium at the USDA, ARS, ABADRU, Manhattan, KS. Twelve mice of each group were intranasally inoculated with 1.58×10^6 TCID₅₀ of the MP12 virus in a volume of 70 μ l (35 μ l per nostril) under anesthesia using isoflurane. The remaining two mice of each group served as negative controls. Mice were weighed every 2 days and observed daily for clinical symptoms. On 3 and 6 days post-inoculation (dpi), three inoculated mice and one negative control from each group were euthanized and necropsied. The remaining six mice of each group were kept for 14 days. If a mouse has more than 25% weight loss or showed signs of neurologic disease, it was humanely euthanized and necropsied. During necropsy, livers, brains, spleens, and blood from each mouse were collected under sterile conditions. The half of liver or brain from each mouse collected and stored at -80°C for later virus detection, and the part of these tissues and spleen was fixed in 10% formalin for histopathologic analysis (C. Liu et al., 2011). Tissues were homogenized twice in PBS for 1 min in a Mini BeadBeater-8 (Biospec Products) to make 10% tissue homogenates. The homogenate was centrifuged at 640 g for 5 min, and the supernatant was transferred to 1.5 ml reaction tubes for virus isolation and titration in Vero E6 cells as described previously (Richt et al., 2003). The serum was isolated from collected blood samples for further viremia analysis using the real-time RT-PCR assay.

RNA Extraction and Real-time RT-PCR

Total RNA was extracted from serum samples collected from each mouse using the Applied Biosystems MagMAX-96 total RNA Isolation Magnetic bead capture kit according to the manufacturer's protocols. Briefly, 130 μ l of lysis/binding buffer was mixed with 50 μ l of sera followed by shaking in a 96-well plate. Then 20 μ l bead mix was added and the mixture was shaken for 5 min. After washing (150 μ l each) for four times, the RNA was eluted in 50 μ l of elution buffer at 65°C. RNA was quantitated using UV spectroscopy.

Real-time RT-PCR was performed to detect viral RNA of serum samples using the AgPath ID rRT-PCR Kit (Life Technologies, Inc., Grand Island, NY) as described previously (W. Wilson et al., 2013). Briefly, the RVFV triplex rRT-PCR procedure was designed to contain L, M and S primers and probes and one of the external RNA control combinations using the following cycling conditions: 45°C for 10 min, 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 1 min. The Ct value of at least two of the three RVFV segments from one sample below 35 cycles would be considered positive.

Histopathological and Immunohistochemical Analysis

Tissues including liver, brain, and spleen in 10% formalin buffer were routinely processed; sections were cut at 4 microns thick on a microtome and stained with hematoxylin and eosin. The amount of necrosis and inflammation were graded macroscopically. In the spleen and liver samples, a 5 point scale was used: 0=No necrosis, 1=Occasional scattered single cell necrosis, 2=Scattered 0.5-2 mm foci of random necrosis, the 3=Scattered larger random area of necrosis, often coalescing with each other, 4=Diffuse lobular necrosis. In the spleen, necrosis was seen as lymphocytolysis in periarteriolar sheaths. In the brain samples, a 4-point scale was

used: 0=No necrosis, 1=one focal area of necrosis and inflammation, 2=2-5 areas of necrosis and inflammation, 3≥6 areas of necrosis and inflammation.

For immunohistochemical analysis, tissues were deparaffinized and rehydrated, antigen retrieval was performed in pH 9.0 EDTA buffer, Bond ER Solution 2 (Leica Microsystems, Buffalo Grove, IL) for 20 minutes at 100° C on the Leica Bond-Max autostainer. The primary polyclonal rabbit anti-RVSV antibody (Drolet et al., 2012) was diluted 1:3000 in Bond Primary Antibody Diluent (Leica Microsystem) and incubated for 15 minutes at ambient temperature with the tissue on the slide. Polymerization was done with Anti-rabbit Poly-HRP-IgG (Leica Microsystems, Buffalo Grove, IL) for 25 min at ambient temperature. Tissues were blocked with 3% hydrogen peroxide for 5 minutes, visualized with DAB, and counterstained with hematoxylin. Immunohistochemical positivity was graded as follows: 0=No staining, 1=Scattered individual cell staining, not more than 15 cells, 2=Moderate numbers of scattered cells, and 3=Diffuse multifocal staining, severe, greater than 75% of the sectional area with staining.

Statistical analysis

The student t-test was used to analyze differences between groups for data collected at a given time point. A $p \leq 0.05$ was considered statistically significant.

2.3 Results

Clinical symptoms, morbidity and mortality of different mouse strains infected with the RVSV MP12 virus

RVSV can be transmitted to human by aerosol exposure and intranasal infection with RVSV induces earlier and more severe neuropathology in the mouse model compared with other infection routes (Reed et al., 2013). Therefore, six different mouse strains of 12 mice/group were

intranasally inoculated with 1.58×10^6 TCID₅₀/mouse of the RVFV MP12 virus in this study. Two control mice euthanized on day 3 and 6 dpi in each group did not show any clinical symptoms and gained weight normally. All infected STAT-1 KO mice started to exhibit weight loss at 2 dpi (Figure 6A) and obvious clinical signs including decreased activity, huddling, hunched posture, and ruffled fur as early as 4 dpi. Three STAT-1 KO mice were humanely euthanized as they developed neurological symptoms such as continual cage circling and head pressing at 8 (1 mouse) and 10 dpi (2 mice), and their weight loss was also close to 25%. The remaining 3 mice began to gain weight at 10 dpi but did not recover until the end of the experiment (14 dpi). In contrast, the BALB/c and C57BL/6J mouse strains started to gain weight post inoculation of RVFV MP12 virus and did not show clinical signs except for 2 BALB/c mice that developed vestibular neurological symptoms (continual cage circling and head pressing) at end of the study (14 dpi) and were euthanized. The infected NZW/LacJ mice showed a slight weight loss at 2 dpi and a severe weight loss at 12 dpi, then started to recover (Figure 6A). No obvious clinical signs were observed in these infected mice. Both infected 129S1/SvImJ and 129S6/SvEv mice showed approximately 10% weight loss at later time points (8 dpi or 10 dpi) when they displayed clinical signs such as decreased activity and ruffled fur. One of 6 infected 129S1/SvImJ mice showed neurological symptoms at 8 dpi and was humanely euthanized. In summary, RVFV MP12 infection caused weight loss and clinical signs in 3 mouse strains including STAT-1 KO, 129S1/SvImJ and 129S6/SvEv, but the STAT-1 KO mice developed more severe disease with 50% mortality than other tested mouse strains (Figure 6A and 6B).

Viral replication in mouse livers and brains

Three infected and one negative control mice from each group were sacrificed on 3 and 6 dpi and viral titers of their livers and brains were determined. Virus was not detected in both

liver and brain samples from control mice of all strains on either day. Virus was detected in liver samples of each infected mouse strain on both days (Figure 7A). Virus titer of liver samples in infected STAT-1 KO mice on 3 dpi was higher than those of other 5 mouse strains. The virus titer in liver of the 129S6/SvEv mice was significantly lower than those of the NZW/LacJ and C57BL/6J mouse strains (Figure 7A), but the C57BL/6J mice did not show clinical symptoms. Although a higher virus titer was detected in the liver of the 129S1/SvImJ on 6 dpi, no significant difference was observed among groups (Figure 7A). Virus was recovered from the brains of each infected mouse strain on both days, but statistically significant lower titers were found in both NZW/LacJ and C57BL/6J mouse strains when compared to the other 4 mouse strains (Figure 7B). Furthermore, a significant lower virus titer was detected in brain samples of infected 129S1/SvImJ mice when compared to those in STAT-1 KO, 129S6/SvEv and BALB/c mice at 3 dpi (Figure 7B). The results indicated that the RVFV MP12 was able to infect each tested mouse strain via the intranasal infection, but it showed different replication in the mouse livers and brains.

Immunohistochemistry results

To detect RVFV MP12 antigens, immunohistochemistry staining was performed for the tissues including liver, brain, and spleen collected from mice euthanized on 3 and 6 dpi and from those that were euthanized later due to neurological symptoms (Table 3). In the liver, most RVFV MP12 antigen detected was seen in the STAT-1 KO group (Table 3, Figure 9B) with 3 mice euthanized on 3 dpi and two mice euthanized later. One 129S6/SVEV mouse and one 129S1/SVLMJ mouse had RVFV MP12 antigen detected in the liver. RVFV MP12 antigen detected was seen in the spleen of STAT-1 KO mice that were euthanized at 3 dpi (Figure 9D) or euthanized later due to neurological symptoms. One BALB/c mouse and two 129S6/SvEv mice

had RVFV MP12 antigen detected in the spleen at 3 dpi (Table 3). RVFV antigen detected was also present in the brain of 3 STAT-1 KO mice (Table 3, Figure 9F), 2 BALB/C mice, and one 129S1/SvImJ mouse that were euthanized later due to neurological symptoms. No RVFV MP12 antigen was detected in the brain of all mice euthanized on 3 dpi (Table 3).

Immunohistochemistry scores were summarized in Table 3.

Viremia of serum samples collected from infected different mouse strains

Real-time RT-PCR assay was performed to detect viral RNA in serum samples collected from inoculated mice that were necropsied on 3 and 6 dpi and those that displayed neurological symptoms were humanely euthanized. Viral RNA was detected in all samples from STAT-1 KO mice on 3 and 6 dpi, but not in all 3 samples collected from humanely euthanized on 8 and 10 dpi (Table 1), indicating that the virus was cleared from blood at later time points. All samples collected from infected NZW/LacJ and C57BL/6J mice on both days were negative. One of 3 serum samples from infected 129S6/SvEv group on 3 dpi was positive; whereas the infected 129S1/SvImJ group had 2 positives out of 3 samples on 6 dpi (Table 1). Viral RNA was detected in serum sample of one mouse of infected BALB/c group on 6 dpi, and in one of 2 humanely euthanized animals on 14 dpi (Table 1).

Microscopic lesions of tissues collected from infected different mouse strains

Liver, spleen and brain collected from each infected and control mouse were examined for lesions and scored. Five out of 6 groups of mice, which include the STAT-1 KO, BALB/c, 129S6/SvEv, C57BL/6J and 129S1/SvImJ, had histopathological lesions in one or more organs when compared to each respective control animals. Lesions were similar between groups but varied in severity. Histopathological scores for mice are summarized in Table 2. In the liver, there were random foci of hepatocellular necrosis and apoptosis with small numbers of

inflammatory cells, which included neutrophils, Kupffer cells and a few lymphocytes (Figure 8B). In the spleen, 3 STAT-1 KO and 1 129S1/SvImJ mice had mild lymphocytolysis in the white pulp (Figure 8D) and 1 STAT-1 KO mouse had a few individual scattered cells in the red pulp. The remainder of the mice in this study had normal spleens, which did not have necrosis or inflammation. Mice in this study had mild lymphoid hyperplasia and extramedullary hematopoiesis in the spleen in both control and infected animals of each strain; these findings were interpreted as incidental in the mouse splenic tissues and the role RVFV infection played in the development of hyperplasia cannot be determined. Lesions in the brain were consistent with varying degrees of encephalitis that included areas of pyknotic and karyorrhectic cellular debris with gitter cells and neutrophils. In addition, vessels were cuffed by neutrophils, lymphocytes and plasma cells (Figure 8F). On histopathological exam, three STAT-1 KO mice, which were humanely euthanized at 8 or 10 dpi due to neurological symptoms, had the most severe lesions in the brain, followed by the liver and then the spleen. Two BALB/C mice, which were humanely euthanized at 14 dpi, also had lesions in the brain. In the mice scheduled to be euthanized on 3 dpi, lesions were observed in both the liver and spleen in STAT-1 KO and in the liver of the BALB/c group. The infected groups of BALB/c, 129S6/SvEv, C57BL/6J and 129S1/SvImJ on 6 dpi had liver lesions. Both 129S6/SvEv and 129S1/SvImJ groups had lymphocytolysis in the spleen on 6 dpi while 129S6/SvEv was the only one to have encephalitis in 6 dpi.

2.4 Discussion

The aim of this study was to establish a mouse model susceptible to infection with RVFV MP12 virus that can be used in the development and evaluation of antiviral therapeutics in BSL-2 facilities in future studies. This mouse model using RVFV MP12 that can be used in a BSL-2 level facility will make RVF research more affordable and facilitate identification of effective

antivirals against RVFV for human and animal use. Our results showed different susceptibilities of six mouse strains to RVFV MP12 vaccine strain infection based on clinical symptoms, mortality, viremia, virus replication, histopathological and immunochemistry analysis. The STAT-1 KO mice were the most susceptible to the tested six mouse strains, evidenced by that infected STAT-1 KO mice displayed obvious clinical signs including weight loss and neurological disease, 50% mortality, viremia, virus replication and histopathological lesions detected in liver and brain. Especially, the infected STAT-1 KO mice developed both acute-onset hepatitis and delayed-onset encephalitis that is very similar to the severe consequences of human RVFV infection (D. Smith et al., 2010). In contrast, both 129S1/SvImJ and 129S6/SvEv mouse strains showed slight weight loss and clinical signs at later time points. Other three mouse strains including C57BL/6J, NZW/LacJ, and BALB/c did not show any clinical signs; however, the virus was detected in livers and brains of both NZW/LacJ and BALB/c strains, suggesting that they are not resistant to infection. Previous studies have reported that NZW/LacJ, 129S1/SvImJ, and C57BL/6J mice are susceptible to infection with Punta Toro virus with approximately 100% mortality, another phlebovirus in the *Phenuiviridae* family (Ashley et al., 2011; Mendenhall et al., 2009); however, they are not susceptible to infection of RVFV MP12 strain. This fact suggests that different *phleboviruses* show differential pathogenicity in the same mouse strain.

Although the 129S6/SvEv mice are the genetic background of STAT-1 KO mice, they are not as susceptible to RVFV MP12 infection when compared to the STAT-1 KO mice. The STAT-1 KO mice have a deficient immune cell response to alpha (α) and gamma (γ) interferons (Meraz et al., 1996), resulting in failure to induce type I IFN response and enhanced susceptibility to MP12 infection. Previous studies also showed that that a low dose of the MP12 strain (approximately 10^4 PFU) can cause 100% mortality in Sv129 IFN α/β receptor $-/-$ mice

that are defective for the IFN- α/β receptor (Boshra, Lorenzo, Rodriguez, et al., 2011; Bouloy et al., 2001; Lorenzo et al., 2010); whereas the Sv129 IFN γ receptor-deficient mice, which are defective for the IFN γ receptor, did not show any disease symptoms when infected with a same dose of the MP12 virus (Bouloy et al., 2001). It has been shown that MBT/Pas mouse cells induced an impaired induction of type I IFN response, leading to its extreme susceptibility to infection with the virulent RVFV ZH548 and Kenya 98 strains compared with BALB/cByJ mice (Do Valle et al., 2010). All these data indicate that the IFN response plays an important role in controlling RVFV infection, but IFN- α/β is absolutely required when compared to the IFN- γ . BALB/c mice are susceptible to infection with the virulent RVFV (Mendenhall et al., 2009; D. Smith et al., 2010) and die due to severe hepatitis and encephalitis after infection (D. Smith et al., 2010). However, our results showed that they are not susceptible to RVFV MP12 strain infection although several BALB/c mice developed similar neurologic symptoms at the end of the experiment as those observed in the infected STAT-1 KO mice. The neurologic symptoms observed in BALB/c mice most likely attribute to the intranasal inoculation as a previous study has reported that aerosol exposure to RVFV induces earlier and more severe neuropathology in the mouse model when compared to the subcutaneous exposure (Reed et al., 2013). Aerosol exposure of wild-type RVFV results in uniformly fatal disease in mice, which is similar to subcutaneous infection with the same virus (Brown, Dominik, & Morrissey, 1981; Reed et al., 2013). Furthermore, RVFV can be transmitted to humans by aerosol exposure, which is evident by several laboratory workers who have become infected (Smithburn, Mahaffy, Haddow, Kitchen, & Smith, 1949). This indicates the potential high risk of infection of veterinarians and abattoir workers who handle infected animals. In our study, the STAT-1 KO mice developed acute-onset hepatitis and delayed-onset encephalitis after intranasal infection with attenuated MP12 vaccine strain, which

is consistent with the fatal neurological disease of C57BL/6 mice intranasally infected with an attenuated RVFV lacking NSs (Dodd et al., 2014). The neurologic disease is most likely associated with the intranasal infection, not due to immune-mediation (Dodd et al., 2014), which is consistent with findings in infected BALB/c and 129S1/SvImJ mice that also developed neurologic symptoms in our study.

Histopathological lesions and RVFV antigen detected were seen in the STAT-1 KO, 129S6/SvEv, BALB/c, C57BL/6J, and 129S1/SvImJ, which means these mice strains have varying degrees of susceptibility to RVFV MP12. Lesions in all groups of mice in this study were consistent with those that have been previously published and included hepatic necrosis with neutrophils, lymphocytolysis in the spleen, and multifocal encephalitis in a BALB/c mouse model challenge with RVFV strain ZH501 (D. Smith et al., 2010). All control and infected mice had lymphoid hyperplasia in the spleen which is found in normal mice and can depend on the location of the lymph node, age, health status and plane of section(Elmore, 2006). Since control mice were negative by IHC test, the lymphoid hyperplasia of the spleen was not interpreted as a lesion of rift valley fever infection. The most severe lesions, histopathological scores, and RVFV antigen detected were present in the STAT1-KO mice which coincides with increased mortality and weight loss supporting that STAT1-KO mice are a good mouse model for MP12 infection.

Despite the STAT-1 KO mouse strain exhibiting severe diseases post infection with the RVFV MP12 strain, it is still not an ideal model to evaluate vaccine efficacy as it is immunodeficient, leading to failure to induce host type I IFN responses against virus infection. However, it could be a very useful small animal model at the BSL-2 level facility to initially screen and elevate antiviral compounds against RVFV prior to using virulent RVFV strains in a high biocontainment facility.

In conclusion, the STAT-1 KO mouse strain is susceptible to MP12 virus infection and develops representative disease including acute-onset hepatitis and delayed-onset encephalitis, indicating that STAT-1 KO mouse model for the MP12 has potential to be used to develop and evaluate RVF antivirals in a BSL-2 environment.

2.5 Conclusion

The STAT-1 KO mouse strain is susceptible to MP12 virus infection and develops the disease. The STAT-1 KO model for the MP12 has the potential to be used to investigate antivirals in a BSL-2 environment.

Table 1 Viremia test results based on real-time RT-PCR.

Mouse strain	3 dpi	6 dpi	Early humane euthanasia
STAT-1 KO	3/3 ^a	3/3	0/3
129S6/SvEv	1/3	0/3	N/A ^b
NZW/LacJ	0/3	0/3	N/A
BALB/c	0/3	1/3	1/2
C57BL/6J	0/3	0/3	N/A
129S1/SvImJ	0/3	2/3	0/1

a. Positive animals/total animals; b. Not applicable.

Table 2 Histopathological scores for scheduled euthanized mice and early humane euthanized mice.

	Scheduled Euthanasia						Early Humane Euthanasia		
	3dpi			6dpi			Liver	Brain	Spleen
	Liver	Brain	Spleen	Liver	Brain	Spleen			
STAT-1 KO	3/3 ^a (1.5±0.5)	0/3	2/3 (1.5±0.5)	0/3	0/3	0/3	2/3 (2.0±0.0)	3/3 (3.0±0.0)	1/3 (1.0)
129S6/SvEv	0/3	0/3	0/3	1/3 (1.0)	1/3 (1.0)	0/3	N/A ^b	N/A	N/A
NZW/LacJ	0/3	0/3	0/3	0/3	0/3	0/3	N/A	N/A	N/A
BALB/c	1/3 (1.0)	0/3	0/3	2/3 (2.0±0.0)	0/3	0/3	0/2	2/2 (1.5±0.5)	0/2
C57BL/6J	2/3 (1.5±0.5)	0/3	0/3	2/3 (1.5±0.5)	0/3	0/3	N/A	N/A	N/A
129S1/SvImJ	0/3	0/3	0/3	3/3 (2.0±0.0)	0/3	1/3 (1.0)	0/1	1/1 (2)	0/1

a. Positive animals/total animals (mean histological score ± absolute deviation); b. Not applicable.

Table 3 Immunohistochemistry scores for scheduled euthanized mice and early humane euthanized mice.

	Scheduled Euthanasia						Early Humane Euthanasia		
	3dpi			6dpi			Liver	Brain	Spleen
	Liver	Brain	Spleen	Liver	Brain	Spleen			
STAT-1 KO	3/3 (2.5±0.5) ^a	0/3	3/3 (2.0±0.0)	2/3 (1.0±0.0)	0/3	0/3	2/3 (1.0±0.0)	3/3 (2.0±0.0)	3/3 (1.5±0.5)
129S6/SvEv	1/3 (1.0)	0/3	2/3 (1.0±0.0)	3/3 (1.0±0.0)	0/3	1/3 (1.0)	N/A ^b	N/A	N/A
NZW/LacJ	0/3	0/3	0/3	1/3 (2.0)	0/3	1/3 (1.0)	N/A	N/A	N/A
BALB/c	0/3	0/3	1/3 (1.0)	1/3 (1.0)	0/3	0/3	0/2	2/2 (3.0±0.0)	0/2
C57BL/6J	1/3 (2.0)	0/3	0/3	3/3 (1.5±0.5)	0/3	1/3 (1.0)	N/A	N/A	N/A
129S1/SvImJ	1/3 (1.0)	0/3	0/3	3/3 (2.5±0.5)	0/3	0/3	0/1	1/1 (3)	0/1

a. Positive animals/total animals (mean histological score ± absolute deviation); b. Not applicable.

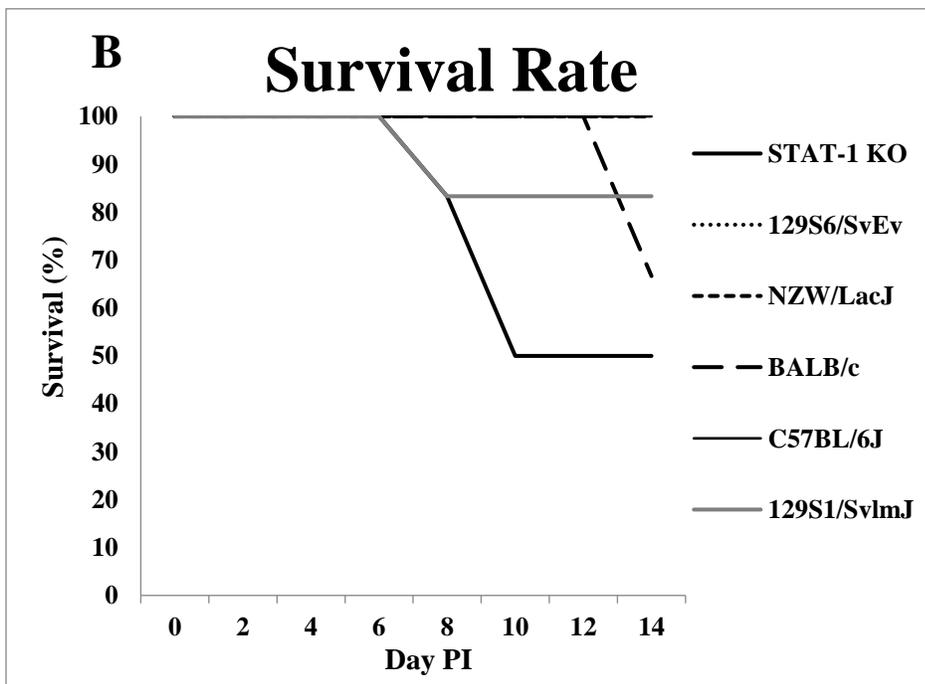
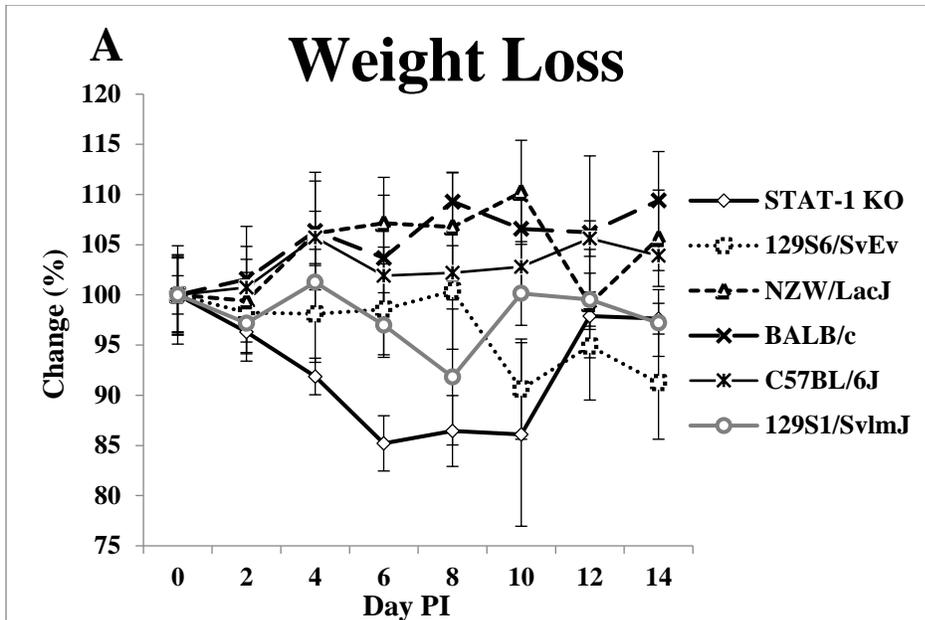


Figure 6 Mouse weight loss and survival rate post infection with the MP12 virus.

Each mouse strain was intranasally infected with 1.58×10^6 TCID₅₀/mouse of the MP12 vaccine strain and was monitored for 14 days post infection. A) Mouse weight loss: the percentage weight from each group and each time point are presented as means \pm SD, and B) Mouse survival rate.

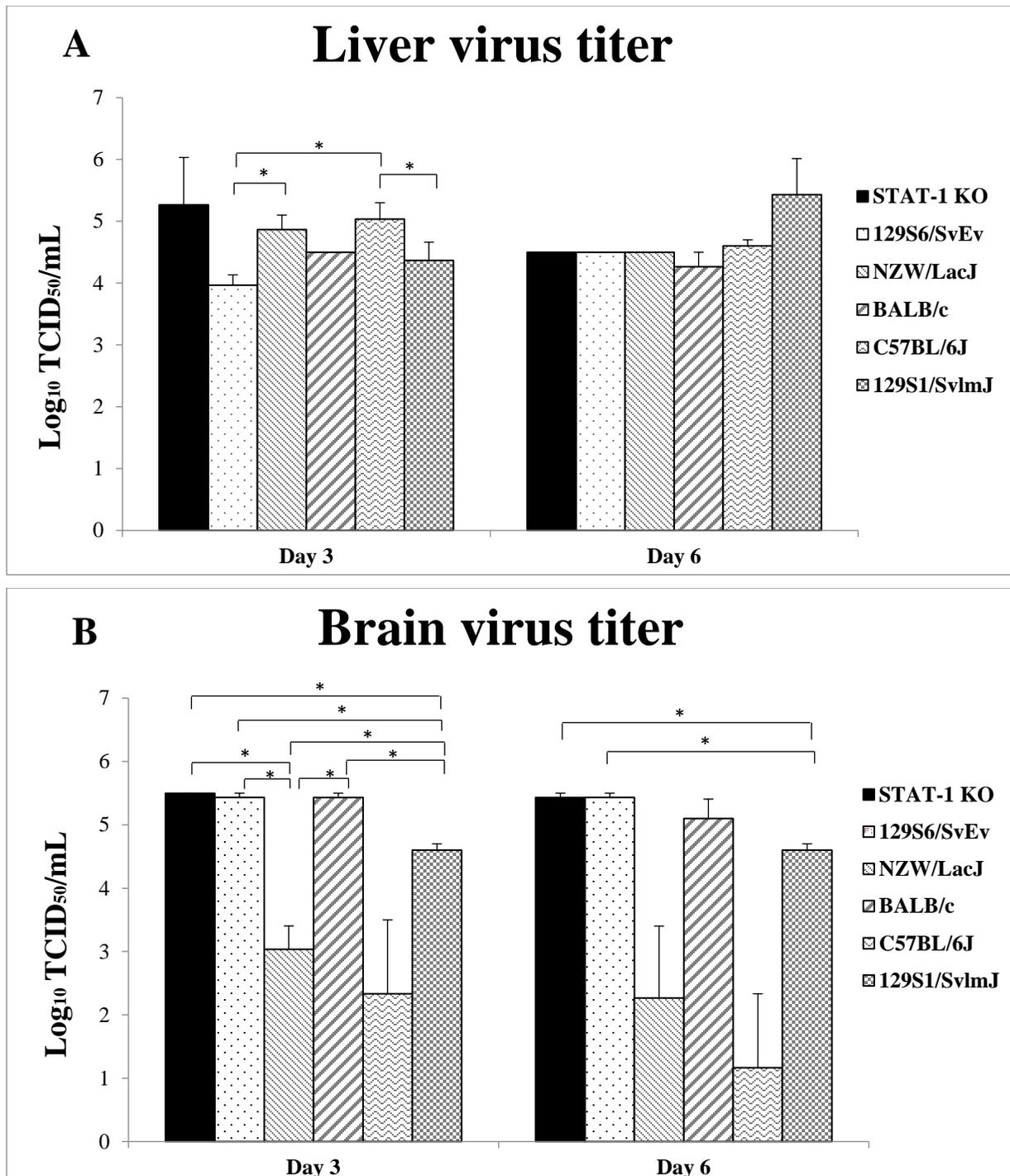


Figure 7 Virus titers of liver and brain samples collected from each mouse infected with the MP12 virus.

A) Virus titer in liver samples, and B) virus titer in brain samples. Three mice from each mouse strain were necropsied at 3 and 6 days post-infection. All animals were positive for virus isolation at indicated time points. Error bars represent \pm SEM (* P < 0.05).

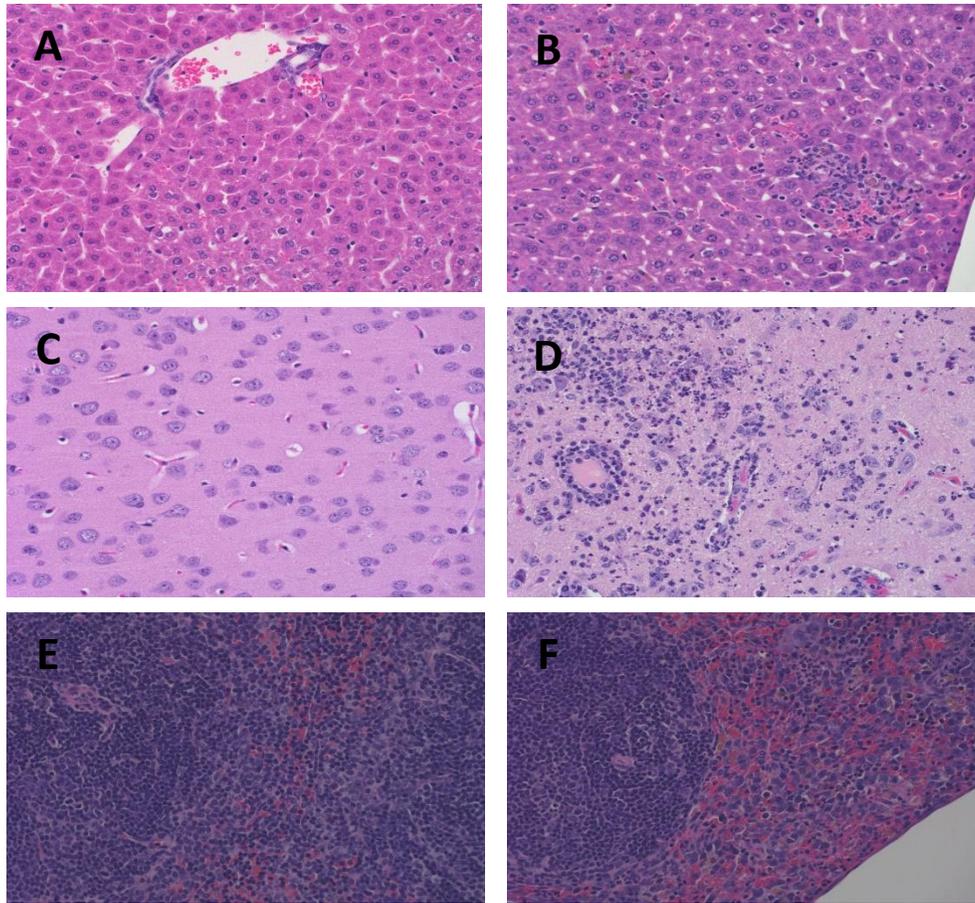


Figure 8 Microscopic liver, brain and spleen sections from control or infected STAT-1 KO mice with the MP12 virus.

A) This liver photomicrograph does not have inflammation or necrosis, which is consistent with normal liver in a control STAT-1 KO mouse that was euthanized at 3-day post infection. B) Liver of an infected STAT-1 KO mouse that was euthanized at 3-day post infection. Two areas of inflammatory cells consisting of viable and degenerate neutrophils, Kupffer cells, and lymphocytes admixed with necrotic and degenerate cells. C) No necrosis or inflammation in the normal spleen of a control STAT-1 KO mouse that was euthanized at 3-day post infection. D) Spleen from an infected STAT-1 KO mouse that was euthanized at 3-day post infection. There are extramedullary hematopoiesis, a lymphoid follicle, and the red pulp. There is no obvious lesions present. Macrophages containing hemosiderin (brown cytoplasmic pigment from the breakdown of red blood cells) are present. E) No necrosis or inflammation consistent with the normal brain of a control STAT-1 KO mouse that was euthanized at 6-day post infection. F) The brain of an infected STAT-1 KO mouse showed neurological symptoms and euthanized at 8-day post infection. Encephalitis was characterized by necrosis and cellular debris. An inflammatory infiltrates composed of degenerate and intact neutrophils, macrophages and lymphocytes, which also cuff vessels. Neuronal necrosis and astroglyosis are present. The magnification of each image is 400 \times .

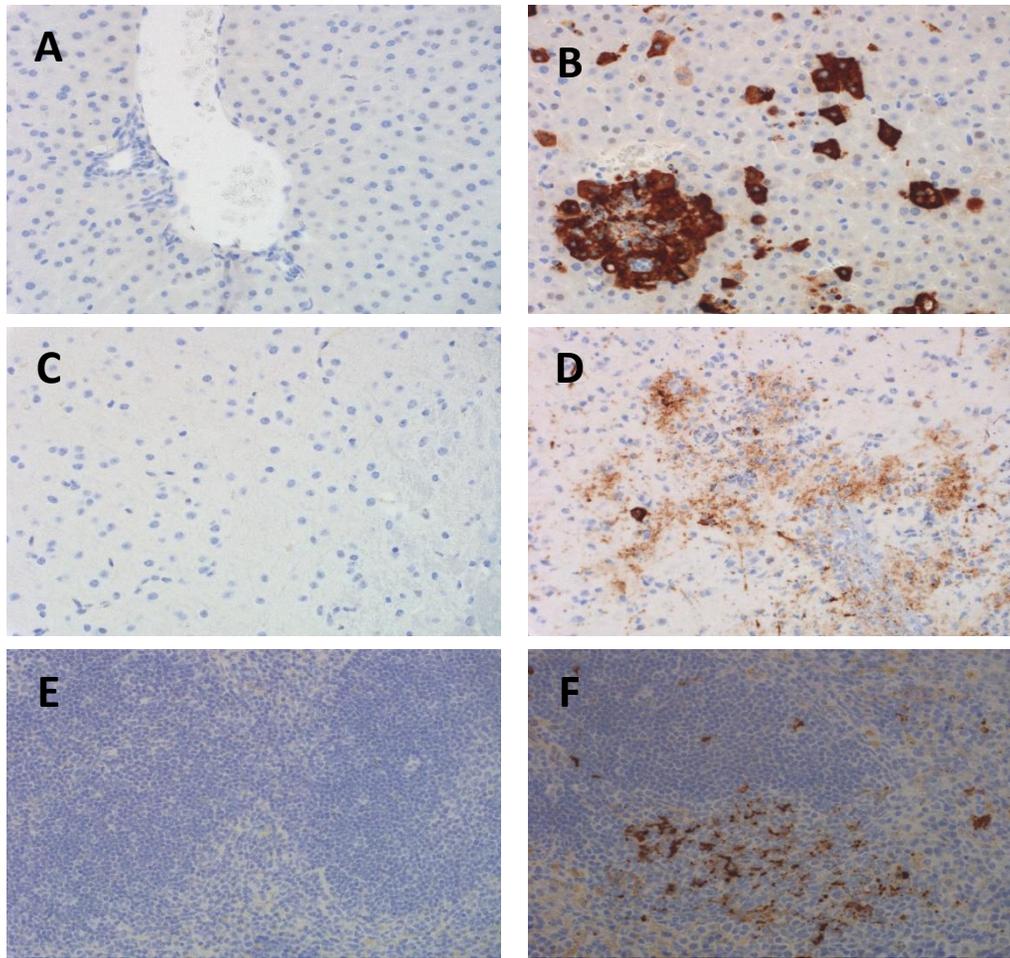


Figure 9 Immunohistochemistry staining results of liver, brain, and spleen sections from control or infected STAT-1 KO mice with the MP12 virus.

A) No RVFV MP12 antigen was present in the liver of a control STAT-1 KO mouse that was euthanized at 3-day post infection; B) Liver of an infected STAT-1 KO mouse that was euthanized at 3-day post infection. Foci of degenerate and viable hepatocytes have positive cytoplasmic RVFV labeling. C) No RVFV MP12 antigen was present in the spleen of a control STAT-1 KO mouse that was euthanized at 3-day post infection. D) Spleen from an infected STAT-1 KO mouse that was euthanized at 3-day post infection. Scattered cells in the lymphoid follicle positive and there are is a group of several cells in the red pulp with positive cytoplasmic labeling. E) No RVFV MP12 antigen was present in the brain of a control STAT-1 KO mouse that was euthanized at 6-day post infection. F) The brain of an infected STAT-1 KO mouse showed neurological symptoms and euthanized at 8-day post infection. Scattered RVFV MP12 antigen among necrotic cells that have ruptured releasing antigen into the neuropil and in the cytoplasm of neurons. The magnification of each image is 400 \times .

Chapter 3 - Identification and evaluation of antivirals for Rift Valley

fever virus

3.1 Introduction

Rift Valley fever virus (RVFV) is the etiologic agent of Rift Valley fever (RVF) that affects both livestock and humans. RVFV is an arbovirus belonged to the genus *Phlebovirus* of the *Phenuiviridae* family. The first documented case was in the Rift Valley of Kenya in 1930 (Daubney et al., 1931). Abortion storm was observed in sheep along the shores of Lake Naivasha during the outbreak (Daubney et al., 1931). Since then, countries in Africa have suffered from numerous RVF outbreaks (Digoutte & Peters, 1989; Gear et al., 1951; JM Meegan, 1979). Although some outbreaks have also been registered in Madagascar in the 1990s, evidence showed that they have resulted from repeated introductions of virus from mainland Africa (Carroll et al., 2011). Around 2000 in Yemen and Saudi Arabia, RVFV emerged for the first time outside Africa in the Arabian Peninsula and caused a dramatic loss of livestock production and fatalities in humans (Balkhy & Memish, 2003; Shoemaker et al., 2002). During 2006-2007 in Somalia, Kenya, and Tanzania, a large RVF epizootics and epidemics occurred and resulted in 315 human deaths out of 1,062 cases, along with the loss of livestock (WHO, 2007). The first imported RVFV human case in China was reported in 2016 (J. Liu et al., 2017). RNA was extracted from the blood sample of the infected patient who returned from Angola, a non-epidemic county, and whole-genome sequencing followed by phylogenetic analysis revealed that a new reassortant from lineage E (L and M segments) and from lineage A (S segment) of RVFV (J. Liu et al., 2017). Although the spread of RVFV outside Africa is mainly due to the commercial transport of infected animals and windborne movement of infected mosquitoes, the facts still raise the concern that the RVF could spread further to the other places in the world.

Until now, neither effective antiviral drugs nor commercial, fully licensed vaccine in the U.S. for human or animal use are available (Eddy et al., 1981; Hunter et al., 2002; Ikegami, 2012; L. Liu et al., 2008). The phylogenetic evidence demonstrated that all strains of RVFV remain closely related (Bird, Khristova, et al., 2007; Grobbelaar et al., 2011; Ikegami, 2012), indicating that antivirals effective to one strain should be sufficient to all RVFV strains. The only conditionally licensed MP12 vaccine strain was derived from a virulent strain ZH548 in the presence of 5-fluorouracil after 12 serial passages (Caplen et al., 1985; Ikegami et al., 2015). Totally 23 mutations were found in the three genome segments and resulted in amino acid substitutions in nine locations including three silent mutations (Lokugamage, Freiberg, Morrill, & Ikegami, 2012). A reassortant study between MP12 and a virulent strain demonstrated that each segment of MP12 is independently attenuated (Saluzzo & Smith, 1990). In 2015, the Ikegami T. group approved that mutations within the M and L segments play more critical roles in the attenuation of the MP12 compared to those in the NSs gene (Ikegami et al., 2015).

To identify the potential antiviral compounds, we employed a cell-based assay using the RVFV rMP12-rLuc strain to screen two NIH Clinical Collections total of 727 compounds, followed by confirmation of inhibition effect on the rMP12-GFP strain. RVFV rMP12-rLuc and rMP12-GFP strains were previously designed using reverse genetics system in a biosafety level 2 (BSL-2) lab (Ikegami et al., 2006). RVFV rMP12-rLuc and rMP12-GFP strains are recombinant MP12 strains which have luciferase/GFP ORF instead of NSs ORF and can express luciferase/GFP stably (Ikegami et al., 2006). Cytotoxicity of the candidates was tested in different concentrations and plaque reduction assay was performed using MP12 parental strain. The 50% inhibitory concentrations (IC_{50}) were further determined using MP12 as well.

The two candidates which can inhibit RVFV replication effectively with limited cytotoxic effect *in vitro* were further evaluated *in vivo* in the STAT-1 KO mouse model. The newly developed STAT-1 KO mouse model is susceptible to MP12 virus infection and develops representative disease including acute-onset hepatitis and delayed-onset encephalitis and can be used to establish and evaluate RVF antiviral compounds in a BSL-2 environment (Lang et al., 2016). Efficacy of the treatments was measured by virus titration (brain and liver), viremia test, and histopathology.

3.2 Materials and Methods

Cells, Virus, and Small molecule Compounds

African green monkey kidney epithelial (Vero) E6 cells (ATCC-CRL 1586) were grown in 1x DMEM medium with 10% fetal bovine serum (FBS, Atlanta Biologicals) (Growth Medium). The RVFV MP12 strain was kindly provided by the U.S. Army Medical Research Institute for Infectious Diseases. The RVFV rMP12-rLuc and rMP12-GFP strains were kindly provided by Dr. Ikegami, at the University of Texas Medical Branch at Galveston. Propagations of the RVFV MP12, the rMP12-rLuc, and the rMP12-GFP strains were done by infecting confluent Vero E6 cells with 0.01 multiplicity of infection (MOI) (Wolf et al., 2010) of the virus in Growth Medium. The viruses were propagated and passaged three times in Vero E6 cells. Cell cultures were collected when 80–95% of the infected cells showed cytopathogenic effect (CPE). The titer of viral stock was determined and calculated as 50% tissue culture infective dose per mL (TCID₅₀/mL) based on the presence of cytopathic effects. Two NIH Clinical Collections total 727 compounds were purchased from Evotec, South San Francisco, CA.

Animals

Seven-week-old female STAT-1 KO mice were obtained from Taconic, Hudson, NY and maintained in a vivarium in the Comparative Medicine Group at Kansas State University, Manhattan, KS. All animal procedures used in this study complied with guidelines set by the USDA and were approved by Kansas State University Animal Care and Use Committee. All efforts were made to minimize suffering.

Time course analysis of different rMP12-rLuc infection doses

For rMP12-rLuc infection, Vero cells were seeded in a 96-well plate till 90% confluence. Then the cells were infected with rMP12-rLuc in triplicate at different MOIs of 0.5, 1.0, 2.0, and 5.0. While three wells of cells were mock-infected with PBS to serve as negative control. After 1 hour post infection (hpi), cells were washed three times with phosphate-buffered saline (PBS) to remove the unattached virus. At 36, 48, and 60 hpi, 20 μ l supernatants of the infected cells in 96-well plates were collected to test the luciferase using Renilla Luciferase Assay (Promega, Madison, WI) following the manufacturer's instruction.

Briefly, cell supernatants were prepared using *Renilla* Luciferase Assay Lysis Buffer prior to the performance of *Renilla* Luciferase Assay (Promega, Madison, WI). Then 20 μ L of cell supernatants were added to the according wells of a black 96-well opaque-bottom plate (Nunc, Rochester, NY), followed by 100 μ l Renilla Luciferase Assay Reagent dispensed to each well. Luminescence measurement of the samples was immediately initiated by a microplate reader (FLUOstar Omega, Cary, NC). Z-factor which is an attempt to quantify the suitability of a particular assay for use in a full-scale, high throughput screen was analyzed to determine the ideal time point and MOI for the high-throughput screening.

Cell-based high-throughput Screening

Total 727 compounds was screened to identify potential RVFV inhibitors. All compounds were dissolved in a final working concentration of 200 μ M and the high-throughput screening was established in a 96-well format. Vero E6 cells were seeded in 96-well plates with 200 μ L Growth Medium in each well. When the cells reached to 90% confluence, 2 MOI RVFV rMP12-rLuc virus was used to infect the cells. After one-hour post infection (hpi), 20 nmol compounds were added to each well respectively. To set up the positive and negative controls, three wells were not infected with RVFV rMP12-rLuc virus or infected with the virus with 20 nmol ribavirin treatment respectively. At 36 hpi, *Renilla* Luciferase Assay (Promega, Madison, WI) was performed as described above. The raw intensity data from each plate were normalized to the same Luminescence (RLU) scale.

Inhibition efficacy test

Vero E6 cells were seeded in 96-well plates with 200 μ L Growth Medium in each well. Then the 90% confluent cells were infected with 2 MOI of RVFV rMP12-GFP. Three wells of cells were not infected with RVFV rMP12-GFP were served as non-treatment control. To set up the positive control, three wells of cells were infected with RVFV rMP12-GFP virus and 20 nmol of ribavirin was given. After one hpi, 20 nmol compounds selected from the high-throughput screening were triplicate added to the plate. Cells were imaged at a 12-hour interval with Nikon Eclipse TE2000-S inverted phase microscope (Nikon, Melville, NY) using excitation/emission wavelength of 470/505 (GFP, green).

Cytotoxicity test

Cellular toxicity was assayed using ToxiLightTM bioassay kit (Lonza, Anaheim, CA) according to manufacturer's instruction. Briefly, 80% confluent Vero E6 cells in 96-well plates

were triplicate treated with 23 compounds which were selected from the first round of screening in 20 or 40 nmol at 37°C for 4 h. Then the cells were washed three times with fresh DMEM followed by adding 100 µL Growth Medium per well. After 2-day incubation, all reagents and culture plate were brought up to room temperature before use. ToxiLight™ 100% lysis reagent set (Lonza) was used to set up the positive control and cells without compound treatment were used as negative control. After 15 min, 20µL of cell supernatant of each well was transferred to a black 96-well opaque bottom plate (Nunc, Rochester, NY) and 100 µL of AKDR was added to each well. After 5 min, the plate was read by a microplate reader (FLUOstar Omega, Cary, NC).

Plaque reduction assay

Vero E6 cells were seeded in 6-well plates with 2 mL Growth Medium in each well. When the cells reached approximately 90% confluence, around 40 plaque forming units (PFU) of RVFV MP12 virus were used to infect the cells in each well, accompany with the 40 nmol candidate compounds. RVFV MP12 infected cells in wells were used to serve as a virus control. Infected cells treated with ribavirin and non-infected cells without treatment were used as negative control and assay control respectively. Agar overlay was added after 1 hpi and the cells were incubated for 4 days at 37 °C followed by staining with 0.4% crystal violet. The compounds which can reduce 50% plaques when compared with that of the virus control were considered as effective antiviral candidates.

Half maximal inhibitory concentration (IC₅₀)

Vero cells were infected with RVFV MP12 strain at 2 MOI in the presence of serially 2-fold diluted mitoxantrone, 6- azauridine or ribavirin from 1000 µM. After 4 hpi, cells were washed three times with fresh DMEM followed by adding 100 µL Growth Medium per well. Supernatants were collected from the cell cultures after 3 days. RVFV infectious dose of each

supernatant was determined by an immunocytochemistry assay. Briefly, Vero cells were infected with 10-fold serially diluted viral supernatant respectively and cultured for 4 days with Growth Medium. After fixation with methanol for 10 min, cells were incubated with a mouse monoclonal antibody against the N protein of RVFV for 1 h. Then, the cells were treated with goat anti-mouse Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP (Invitrogen). TCID₅₀ were determined by the last dilution showing visible specific staining. IC₅₀ were calculated using regression analysis.

Mouse Experiment

Totally 56 mice of seven-week-old female STAT-1 KO mice were maintained in a mouse vivarium in the Comparative Medicine Group at Kansas State University. Mice were divided into four infected groups (13 mice/group) and one control group (4 mice). Mice in each infected group were intranasally inoculated with 1.58×10^6 TCID₅₀ of the MP12 virus in a volume of 70 μ l (35 μ l per nostril) under anesthesia using 4% isoflurane. After one-hour post infection, mice were treated with antiviral drugs twice daily at an 8-hour interval for ten days under light anesthesia with isoflurane. Single doses for ribavirin, 6-azautidine, mitoxantrone were 100 mg/kg, 50 mg/kg, and 0.1 mg/kg respectively. Mice were weighed and observed daily for clinical symptoms. On 3 and 6 dpi, three infected mice from each group and two negative control mice were euthanized and necropsied. The remaining seven mice of each infected group were kept until the end. Mice were euthanized and necropsied if more than 20% of the weight was lost after virus inoculation or showed signs of neurologic disease. During necropsy, samples of liver, brain, spleen, and blood from each mouse were collected under sterile conditions. The half of liver or brain from each mouse were stored at -80°C for later virus detection, and the left part of these tissues and spleen was fixed in 10% formalin for histopathologic analysis (Liu et al., 2011).

Virus detection was performed in a 10% tissue homogenate in PBS that generated by homogenizing twice for 1 min in a Mini BeadBeater-8 (Biospec Products). After centrifuging at 640 g for 5 min, the supernatant of the homogenate was transferred to 1.5 ml reaction tubes for virus isolation and titration in Vero E6 cells as described previously (Lang et al., 2016). The serum was isolated from blood samples for further analysis.

RNA Extraction and Real-time RT-PCR

Applied Biosystems MagMAX-96 Total RNA Isolation Magnetic-bead capture kit was used to extract total RNA from serum samples collected from each mouse following the manufacturer's instructions. Briefly, 50 µl of sera was stirred with 130 µl of lysis/binding buffer. After shaking in a 96-well plate, 20 µl bead mix was added and the mixture was shaken for another 5 min. Then the mixture was washed (150 µl each) for four times. The RNA was eluted in 50 µl elution buffer at 95°C and quantitated by a UV spectroscopy.

As described previously, AgPath ID rRT-PCR Kit (Life Technologies, Inc., Grand Island, NY) was used to perform Real-time RT-PCR to detect viral RNA in serum samples (W. Wilson et al., 2013). Briefly, the RVFV triplex rRT-PCR procedure has been designed to contain L, M and S primers and probes and one of the external RNA control combinations using the following cycling conditions: 45°C for 10 min, 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 1 min. The Ct values with 35 or lower for all three segments was judged as positive.

Histopathological Analysis

The histopathological analysis was performed as described before (Lang et al., 2016). Briefly, tissues including liver, brain, and spleen in 10% formalin buffer were routinely processed. The amount of necrosis and inflammation were graded macroscopically. In the spleen

and liver samples, a 5-point scale was used: 0=No necrosis, 1=Occasional scattered single cell necrosis, 2=Scattered 0.5-2 mm foci of random necrosis, 3=Scattered larger random area of necrosis, often coalescing with each other, 4=Diffuse lobular necrosis. In the spleen, necrosis was seen as lymphocytolysis in periarteriolar sheaths. In the brain samples, a 4-point scale was used: 0=No necrosis, 1=one focal area of necrosis and inflammation, 2=2-5 areas of necrosis and inflammation, 3 \geq 6 areas of necrosis and inflammation.

For immunohistochemical analysis, polyclonal rabbit anti-RVSV antibody (Drolet et al., 2012) was diluted 1:3000 in Bond Primary Antibody Diluent (Leica Microsystem) on selected tissues. Slides were deparaffinized and rehydrated, the antigen was retrieved in pH 9.0 EDTA buffer, Bond ER Solution 2 (Leica Microsystems, Buffalo Grove, IL) for 20 minutes at 100° C on the Leica Bond-Max autostainer. The primary antibody was incubated for 15 minutes at ambient temperature. Polymerization was done with Anti-Rabbit Poly-HRP-IgG (Leica Microsystems, Buffalo Grove, IL) for 25 min at ambient temperature. Slides were blocked with 3% hydrogen peroxide for 5 minutes, visualized with DAB, and counterstained with hematoxylin. Immunohistochemical positivity was graded as follows: 0=No staining, 1=Scattered specific cell staining, not more than 15 cells, 2=Moderate numbers of scattered cells, and 3=Diffuse multifocal staining, severe, greater than 75% of the sectional area with staining.

Statistical analysis

The student t-test was used to analyze differences between groups for data collected at a given time point. A $p \leq 0.05$ was considered statistically significant.

3.3 Results

Time course analysis of different rMP12-rLuc infection doses

The Z-factor is an attempt to quantify the suitability of a particular assay for use in a full-scale, high-throughput screening. The statically ideal Z-factor is 1.0. Between 0.5 and 1.0, the experiment can be considered as an excellent assay. Between 0 and 0.5, the experiment can be considered as a marginal assay. Less than 0, the experiment cannot be used in the high-throughput screening. As shown in Figure 10, data at 36 hpi with different infected doses follow the normal distribution, suggesting data collected at this time point are reliable. While the peak Z-factors is 0.73 at 36 hpi with 2 MOI infection, indicating this assay is excellent for high throughput screening when the observation time point is 36 hpi with the infection dose of 2 MOI (J. Zhang, Chung, & Oldenburg, 1999; X. Zhang, 2008).

High-throughput screening to identify new RVFV inhibitors

Then two NIH Clinical Collections total of 727 compounds were screened. As shown in Figure 11, ribavirin inhibited the rMP12-rLuc replication efficiently. While luminescences of the other tested 23 compounds were statistically significantly different from that of the Mock treatment control, suggesting they might show antiviral effect on rMP12-rLuc and can be used as potential RVFV inhibitors. While the other 704 compounds did not show inhibition effect on RVFV rMP12-rLuc.

Inhibition efficacy and cytotoxicity of the selected 23 potential RVFV antiviral compounds

Since some other factors, such as the interaction between the compound and the Luciferin/ Luciferase, and cell viability, can also result in a reduction of luminescence in the first round of screening, we further tested the inhibition efficacy of these 23 compounds using

rMP12-GFP strain and their cytotoxicity. As shown in Figure 12, Compound 8, 11, 12, 13, 16, 18, 19, 21, and 23 showed less GFP signals compared with the non-treatment cells, indicating that they can inhibit rMP12- GFP effectively. No GFP signal was detected in ribavirin treated cells, indicating it can inhibit the rMP12- GFP replication efficiently. While Compound 11 can inhibit rMP12- GFP as effectively as ribavirin. However, GFP signals were still detectable in Compound 8, 12, 13, 16, 18, 19, 21, or 23 treated cells, indicating they are not as effective as the ribavirin in inhibition of rMP12- GFP.

ToxiLight™ bioassay kit (Lonza, Anaheim, CA) was used to test the cellular toxicity of the 23 compounds with 20 or 40 nmol in 96-well plates. Most of the compounds are toxic to Vero E6 cells at the indicated concentration. This may cause by the compounds are targeting to the cancer cells. Although Vero E6 is a continuous and aneuploid cell line which does not form tumors in immunosuppressed rodents, these cells are not “normal” compared with the primary cells and immortalized cell lines are usually considered as the *in vitro* equivalent of cancerous cells (Sheets, 2000). While two compounds showed limited cytotoxicity also showed inhibition effect on rMP12- GFP. In Figure 13, compared to the toxic and non-toxic controls, Compound 8 and 11 showed no toxicity to Vero E6 cells at the effective antiviral concentrations. Furthermore, intraperitoneal LD₅₀ of Compound 8 in rat is 1640 mg/kg. In addition. Compound 11 showed similar results with Compound 8 and the intraperitoneal LD₅₀ of the agent in the rat is 9400 mg/kg, indicating both of the compounds should be safe to use as antiviral candidates at effective concentrations with limited cytotoxicity. Thus, these two compounds were selected for further test.

Inhibition efficacy of Compound 8 and 11 by plaque assay

Since NSs is the major virulent factor of RVFV and the two recombinant MP12 viruses expressed report proteins instead of NSs, we want to know whether these two candidates can inhibit the replication of the parental strain MP12 or not. Therefore, we did the plaque reduction assay. Compared with the plaque count of the virus control, Compound 8 and 11 treated cells reduced more than 50% plaques indicating they are effective antiviral candidate (Figure 14).

IC₅₀

The antiviral activity of Compound 8 and 11 against the RVFV strain MP12 was evaluated in Vero cells in parallel with ribavirin. Compound 8 and 11 inhibited replication of RVFV by approximately 3 or 7 log units at a concentration of 1,000 μ M (Figure 15), the IC₅₀ of Compound 8 and 11 were 211.4 μ M and 139.5 μ M, respectively. Compared with the IC₅₀ of ribavirin, Compound 11 inhibited the replication of MP12 effectively.

Clinical symptoms, morbidity, and mortality of MP12 infected STAT-1 KO mice with different treatment

The STAT-1 KO mouse model for the MP12 was established and can be used to evaluate antivirals in a BSL-2 environment (Lang et al., 2016). Therefore, mice in four infected groups were intranasally inoculated with 1.58×10^6 TCID₅₀/mouse of the RVFV MP12. Different treatments were given intranasally twice daily at an 8-hour interval for ten days to each infected group after one hour post infection. Four control mice euthanized on 3 and 6 dpi did not show any clinical symptoms and gained weight normally. All mice received placebo or 6-azauridine treatment showed consecutive weight loss starting from 2 dpi till the end. Obvious clinical signs included decreased activity, huddling, hunched posture, and ruffled fur as early as 3 dpi (Figure 16A). In addition, all mice in these two groups reached the 20% weight loss threshold or came to

a moribund condition at 8 dpi (Figure 16B). Noticeable weight loss was observed in ribavirin treated groups at 3 dpi and the mice started to gain weight till 5 dpi. Mice in mitoxantrone treated group exhibited weight loss from 3 dpi and less severe disease was observed overall. However, like the ribavirin treated group, after 5 dpi the weight decreased dramatically till the end (Figure 16A). Ribavirin and mitoxantrone treated mice showed one day delay compared with mice in the other infected groups to reach the 100% mortality (Figure 16B).

Viral replication in mouse livers and brains

Two control and three infected mice from each group were euthanized on 3 and 6 dpi and viral titers in their livers and brains were determined. The virus was detectable in liver samples from each infected mice on both days (Figure 17A). Lower virus titer of liver samples in 6-azauridine treated group ($10 \text{ TCID}_{50}/\text{mL}$) was detected compared with that in the other infected groups, suggesting the 6-azauridine inhibits RVFV replication in liver. Although no significant difference was observed among the placebo, ribavirin, and mitoxantrone treated groups, mice in mitoxantrone treated group showed less severe clinical signs (Figure 17A). MP12 was recovered from the brains of each infected mice at both 3 and 6 dpi. But statistically, significant lower titer was found in 6-azauridine treated mice at 3 dpi when compared to the placebo and mitoxantrone treated mice, suggesting the 6-azauridine inhibits RVFV replication in brain effectively at least for the first 3 dpi (Figure 17B). However, no significant difference was found in titers of brains samples among groups on 6 dpi, indicating MP12 grew dramatically between 3 and 6 dpi in 6-azauridine treated mice.

Viremia of serum samples collected from mice

Real-time RT-PCR was performed to detect viral RNA in serum samples from all infected mice. As shown in Table 4, no viral RNA was detected in all samples collected from

ribavirin treated mice, indicating that ribavirin can inhibit virus replication in blood to an undetectable level. Mice treated with placebo showed viremia only on 3 dpi. While samples collected from the 6-azauridine or mitoxantrone treated mice was positive at different ratios on 3 and 6 dpi and in samples collected from humanely euthanized of infected mice, indicating these two compounds can hardly prevent MP12 from circulating in the blood-stream.

Microscopic lesions and Immunohistochemistry of tissues obtained from mice received different treatment

Livers and brains collected from each mouse were examined for lesions, viral antigen and the summarized scores were shown in Table 5. In the liver, the antigen was detected at both 3 and 6 dpi, sporadic extramedullary hematopoiesis was observed in 6-azauridine treated group at 3 dpi and in ribavirin/mitoxantrone treated group at 6 dpi. No lesions were seen in livers collected from humanely euthanized mice. At 3 dpi, no lesion in the brain was found in each group and only one out of three mice showed antigen positive in the placebo-treated group. Lesions in the brain were observed in all infected groups at 6 dpi but the degrees are various and all humanely euthanized mice showed severe perivascular meningoencephalitis with necrosis (Table 5). As we expected all mice euthanized at 6 dpi with placebo treatment showed encephalitis characterized by necrosis with abundant necrotic cellular debris and meningitis and abundant RVFV antigen labeling was seen (Figure 18I and 18J). While in ribavirin and 6-azauridine treated groups, only one out of three mice have little necrosis in the brain with much less antigen labeling compared with the placebo-treated group at 6 dpi (Figure 18A, 18B, 18E, and 18F). While mice treated with mitoxantrone had moderate RVFV antigen presented in the neurons and the neuropil and the disease was less severe than the placebo-treated group (Figure 18C and 18D).

3.4 Discussion

The aim of this study was to identify antivirals for RVFV from the two NIH clinical collections and evaluate their efficacy *in vitro* and *in vivo*.

Our *in vitro* study screened total 727 compounds using a cell-based high throughput assay and 23 of them showed promising and were selected for further testing. Nine of 23 compounds showed inhibition of the RVFV rMP12-GFP replication; two candidates, 6-azauridine and mitoxantrone, showed efficient inhibition of MP12 replication by the plaque reduction assay with negligible cytotoxicity at the active antiviral concentration.

Mitoxantrone and 6-azauridine were further tested *in vivo* using the previously established mouse model in a BSL-2 environment (Lang et al., 2016). This mouse model can produce representative disease including acute-onset hepatitis and delayed-onset encephalitis. Therefore, routes of treatment administration should be taken into consideration because of the special immune environment of the brain. Intraperitoneal or intravenous administration are easy to perform. But the antivirals into the central nervous system (CNS) needs to penetrate the blood-brain barrier (BBB) which depend on the antiviral characteristics such as molecular size, electric charge, lipophilicity, plasma protein binding, and the host factors (Nau, Sörgel, & Eiffert, 2010). The lack of effective antiviral drugs capable to bypass the BBB is still a problem for therapeutic of RVFV (Bird & McElroy, 2016). Intranasal delivery is a alternative practical method of bypassing the BBB to deliver small therapeutic molecules to the brain and spinal cord (Hanson & Frey, 2008). These therapeutic agents can be delivered to the brain by olfactory and trigeminal pathways which providing a direct connection with the CNS (Dixon et al., 2016; Ross et al., 2004; Thorne, Pronk, Padmanabhan, & Frey, 2004). In addition, the previous study reported that

intranasal application of ribavirin can increase its availability inside the brain (Colombo et al., 2011). So we chose to give the treatment intranasally in order to bypass the BBB.

MP12 infected mice were given different treatments intranasally after one-hour post infection routinely. As we expected, placebo-treated mice showed severe weight loss and first reach the threshold of 20% weight loss with visible neurologic signs at 8 dpi. While ribavirin and mitoxantrone treated mice showed delayed disease compared to the other two groups which exhibited weight loss as early as 2 dpi. Virus was detectable in the blood of all experimental mice except for ribavirin-treated animals. Virus replication was detected in brains and livers of the all experimental mice at both designed necropsy day, but significant lower titer was found in the livers of 6-azauridine treated mice, indicating virus replication was inhibited in the liver in the presence of 6-azauridine. In addition, the significant lower titer was also found in the brains of 6-azauridine treated mice at 3 dpi but not at 6 dpi, demonstrating MP12 can hardly replicate in brains in the presence of 6-azauridine at the early stage of infection. Although 6-azauridine showed inhibition in the mouse livers and brains, it did not improve the outcome compared with the placebo-treated mice.

Histopathological lesions and RVFV MP12 antigen were found in livers and/or brains of mice from each infected group at 6 dpi, but fewer mice and less severe lesions in brains of mice in ribavirin and 6-azauridine treated groups compared with that of the other two groups (Table 5). Virus antigen was detectable in livers of infected mice at 3 dpi but only one mouse in 6-azauridine treated group showed mild lesions. These results indicate that ribavirin and 6-azauridine can inhibit lesion development in brains at least for 6 dpi. No hepatitis was observed but encephalitis in humane euthanasia mice, suggesting the fatal cases are most likely resulted from encephalitis even with antiviral treatment.

In our *in vitro* and *in vivo* studies, we used ribavirin as a positive antiviral drug since it can inhibit RVFV replication (Oestereich et al., 2014). Ribavirin is a broad-spectrum antiviral agent which was first synthesized in 1972 (Snell, 2001). It can hardly penetrate the BBB and subsequently resulting in the failure to prevent delayed-onset neurologic disease. Moreover, a shift in disease characteristics from sudden-onset hepatic disease to delayed-onset neurologic disease was observed in animals which were infected with RVFV and further treated with ribavirin (Peters & Anderson Jr, 1981). Therefore, development of novel compounds with improved safety and efficacy is in urgent need for treatment and control of RVF.

Similar with ribavirin, the mitoxantrone improved the outcome of intranasal MP12 infection in a mouse model. Mitoxantrone is an anthracenedione agent which showed inhibition effect on Poxviruses by blocking the virus assembly (Deng et al., 2007). However, the mechanism of the virus inhibition on Poxviruses is unclear. Vaccinia virus forms protein precursors of structure proteins. In the presence of mitoxantrone, the precursors of vaccinia virus were synthesized during virus infection but limited processing to mature forms (Deng et al., 2007). While RVFV also forms protein precursors of the preglycoproteins, mitoxantrone may employ the same mechanism to inhibit RVFV with vaccinia virus. Deng et al. suggesting that mitoxantrone may target a viral protein because antiviral resistant strains were identified (Deng et al., 2007). However, mitoxantrone showed no beneficial effect *in vivo* against vaccinia virus infection (Altmann et al., 2012; Deng et al., 2007).

Although 6-azauridine did not benefit the survival in RVFV MP12 infected mice, inhibition of the virus replication in lungs and brains was observed. 6-azauridine, a synthetic triazine analogue of uridine with antimetabolite activity, can inhibit multiplication of a broad range of DNA and RNA viruses (Crance, Scaramozzino, Jouan, & Garin, 2003; Neyts,

Meerbach, McKenna, & De Clercq, 1996; Rada & Dragúň, 1977). However, the antiviral mechanism of the 6-azauridine is still unknown. The fact that the RVFV MP12 replicated fast from 3 dpi to 6 dpi in 6-azauridine treated mice may imply that MP12 gained 6-azauridine resistance in the presence of the antiviral.

Our mouse model resembled the severe consequences of human RVFV infection which are acute-onset hepatitis and delayed-onset encephalitis (Lang et al., 2016; D. Smith et al., 2010). But no severe hepatitis was developed in all infected mice in this study. In addition, all infected mice were dead in less than 10 days in this study while the previous study showed 50% mortality after infection. This may result from the more stress received by the mice in the present study, such as twice daily anesthesia and restraining handling, and everyday weighing. Also, mice may be hurt by the side effect from the inhaled dissolved treatment. Therefore, a lot of optimization, such as infection dose, dosage form, formulation, and delivery device, should be done in the future (Vasa, O'Donnell, & Wildfong, 2015).

In summary, two NIH Clinical Collections were screened *in vitro* and two candidates were further tested in the established mouse model in a BSL-2 environment. *In vivo* study demonstrated that only mitoxantrone benefit the outcome in MP12 infected mice. Our results promise better understanding in improving the antiviral development against RVFV. Further studies will focus on the inhibition mechanism of virus replication, and structural analysis and modification of 6-azauridine and mitoxantrone.

3.5 Conclusion

Two antiviral drugs against RVFV were identified and shown to inhibit MP12 replication efficiently *in vitro*. *In vivo* studies testing two candidates, 6-azauridine and mitoxantrone, reveal

that only mitoxantrone has a reduction of virus replication and improved clinical outcomes in mice. These data would benefit antiviral development against RVFV.

Table 4. RNA level in mouse sera determined by a real-time RT-PCR assay

	3 DPI	6 DPI	Humane euthanasia
Ribavirin	-	-	+ (0/7)
6-azauridine	+ (2/3)*	+ (1/3)	+ (1/7)
Mitoxantrone	+ (3/3)	+ (1/3)	+ (1/7)
Sodium chloride	+ (3/3)	-	+ (0/7)

*RVFV RNA positive mouse counts/ total mouse counts

Table 5. Histopathological (HE) and immunohistochemistry (IHC) scores for infected mice with different treatment

	Scheduled Euthanasia								Humane Euthanasia	
	3 dpi				6 dpi				Liver	Brain
	Liver		Brain		Liver		Brain			
HE	IHC	HE	IHC	HE	IHC	HE	IHC	HE	Brain	
Ribavirin	0/3 ^a	2/3 (1.0)	0/3	0/3	1/3 (2.0)	1/3 (1.0)	1/3 (2.0)	3/3 (2.0±1.0)	0/7	7/7 (2.5±0.5)
6-azauridine	1/3 (1.0)	3/3 (1.0)	1/3 (1.0)	0/3	0/3	2/3 (1.0)	1/3 (1.0)	3/3 (2.5±0.5)	0/7	7/7 (2.5±0.5)
Mitoxantrone	0/3	3/3 (1.0)	0/3	0/3	1/3 (1.0)	1/3 (1.0)	3/3 (2.5±0.5)	3/3 (3.0)	0/7	7/7 (3.0)
Sodium chloride	0/3	3/3 (1.0)	0/3	1/3 (1.0)	0/3	2/3 (1.0)	3/3 (2.0±1.0)	3/3 (2.5±0.5)	0/7	7/7 (3.0)

a. Positive animals/total animals (mean histological score ± absolute deviation);

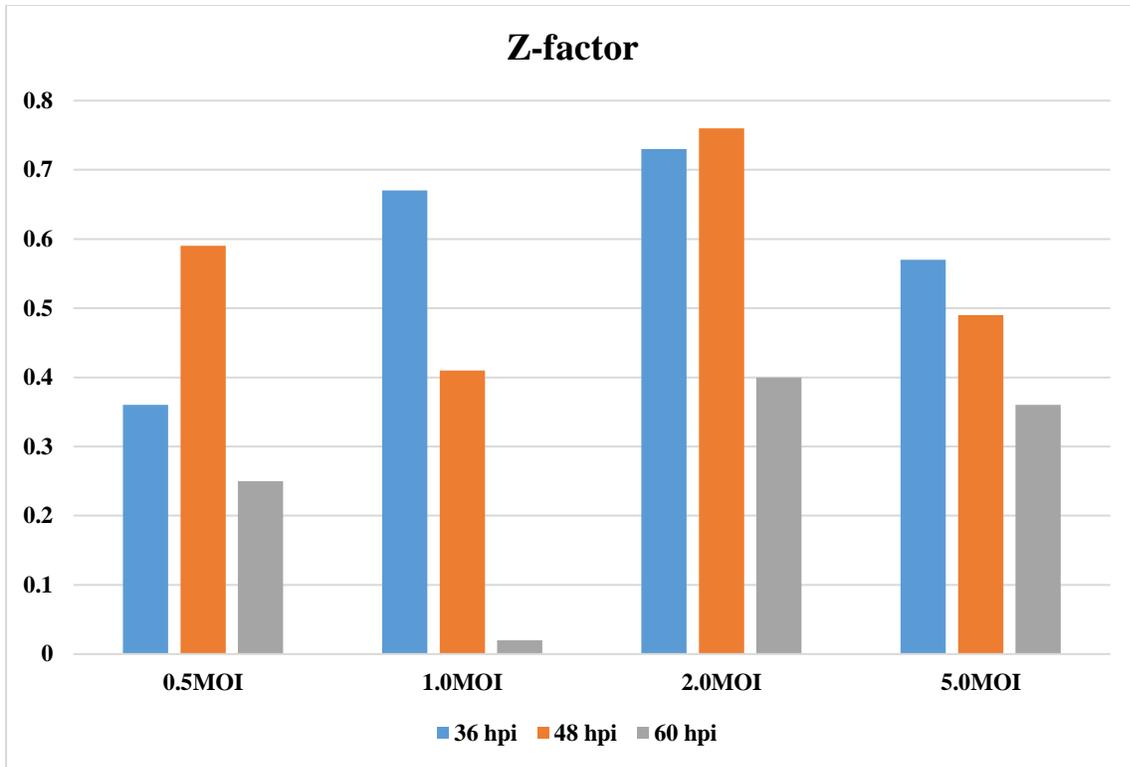


Figure 10 Time course analysis of different rMP12-rLuc infection doses.

The Z-factor is an attempt to quantify the suitability of a particular assay for use in a full-scale, high-throughput screen. Observations at 36 hpi with different infected doses followed the normal distribution. The ideal Z-factor is 1.0. Between 0.5 and 1.0, the experiment can be considered as an excellent assay. Between 0 and 0.5, the experiment can be considered as a marginal assay. Less than 0, the experiment cannot be used in the high-throughput screening.

Renilla Luciferase Assay

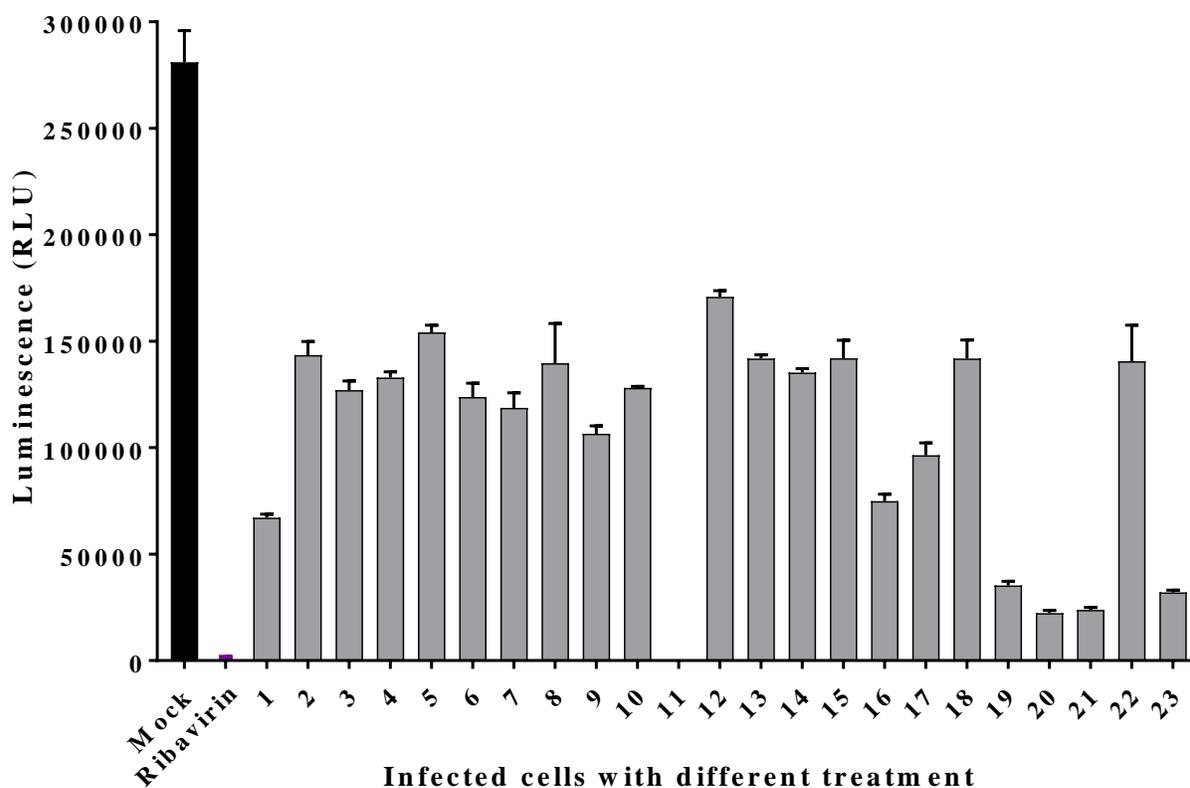


Figure 11 Screening of antiviral compounds by a cell-based high-throughput assay using RVFV rMP12-rLuc strain.

Mock treatment: cells were only infected with rMP12-rLuc without treatment. Ribavirin: cells were treated with ribavirin after infection. Compound 1 – Compound 23: 23 of compounds showed promising results.

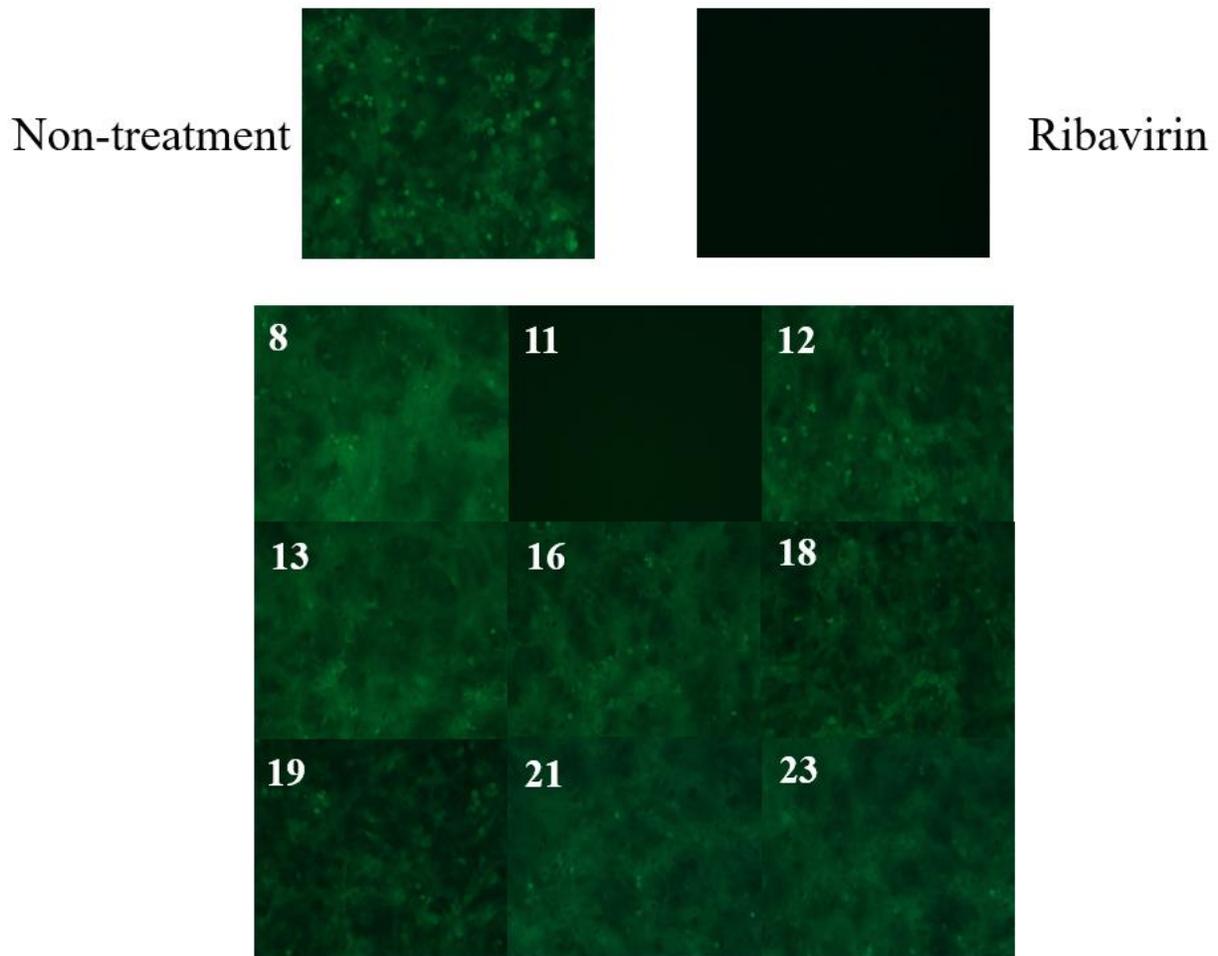


Figure 12 Inhibition efficacy of antiviral compounds by using RVFV rMP12-GFP strain.

Non-treatment: cells were neither infected with virus nor treated. Ribavirin: cells were treated with ribavirin after infection. Compound 8, 11, 12, 13, 16, 18, 19, 21, and 23: Cells were treated with each compound after virus infection. Compound 8 and Compound 11 showed inhibition of the rMP12-GFP virus replication.

Cytotoxicity Assay

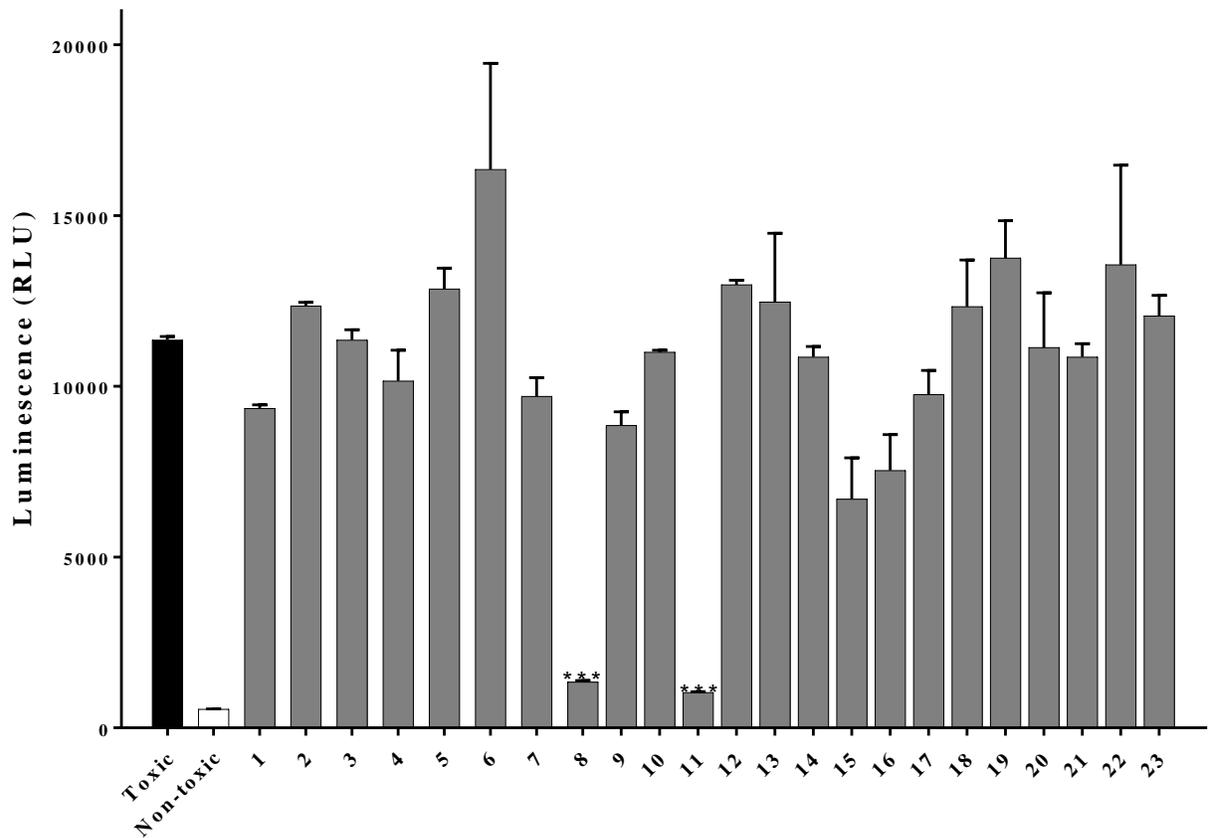


Figure 13 Cytotoxicity test for one representative potential compound with different concentrations.

Toxic: ToxiLight 100% lysis reagent set (Lonza) was used to set up the toxic control. Non-toxic: supernatant from healthy cell culture. Compound 8 (mitoxantrone): Cells were treated with the compound 8 with 40 μ mol. Limited cytotoxicity on Vero E6 cells. Compound 11 (6-azauridine): Cells were treated with the compound 11 with 40 μ mol. Limited cytotoxicity on Vero E6 cells. Error bars represent \pm SEM (***) P < 0.001)

Plaque Reduction Assay

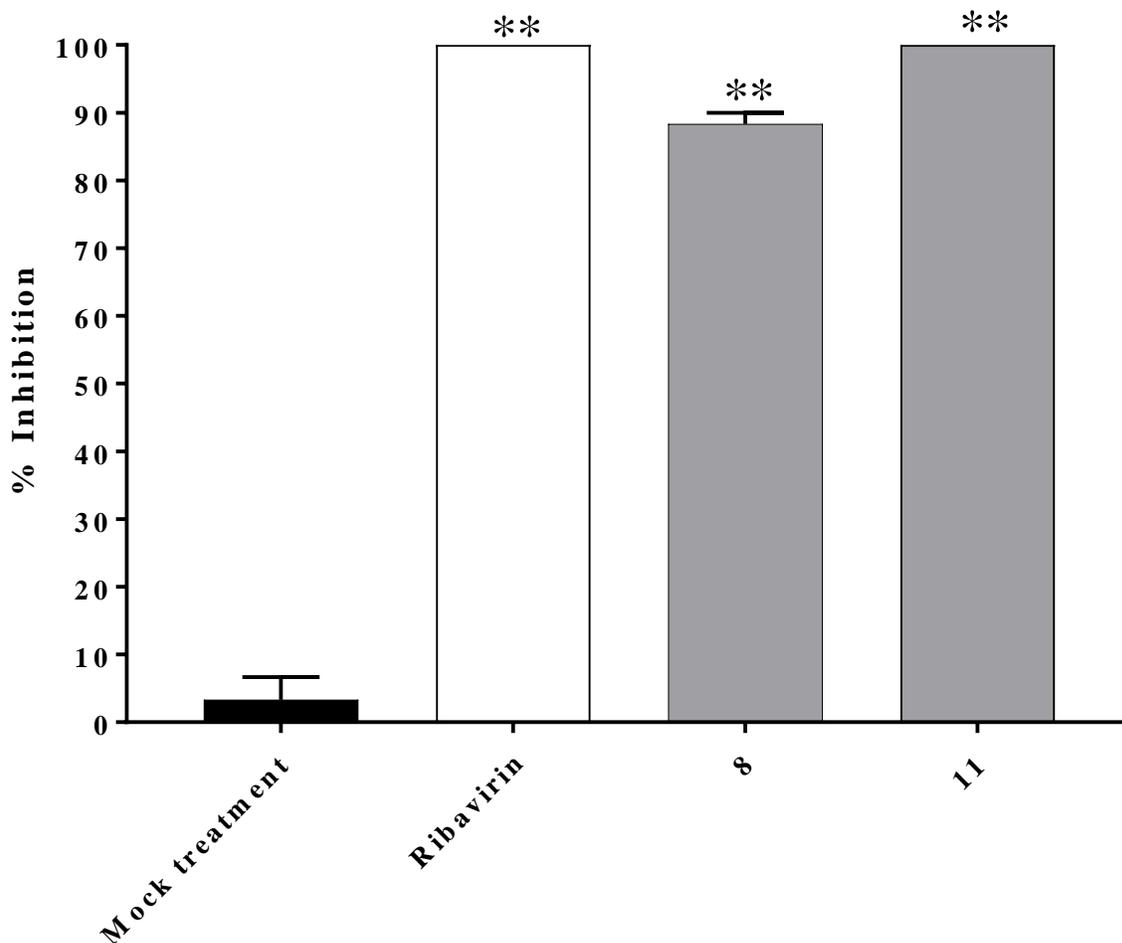


Figure 14 Plaque reduction of different compounds using MP12 strain.

Mock treatment: cells were only infected with MP12 without treatment. Ribavirin: Cells were treated with 40 μmol ribavirin after MP12 infection. Compound 8 (mitoxantrone): cells were treated with 80 μmol the Compound 8 after MP12 infection. Compound 11 (6-azauridine): cells were treated with 80 μmol the Compound 11 after MP12 infection. Compound 8 and 11 showed statically significant plaque reduction effect on MP12 strain. Error bars represent \pm SEM (** P < 0.01)

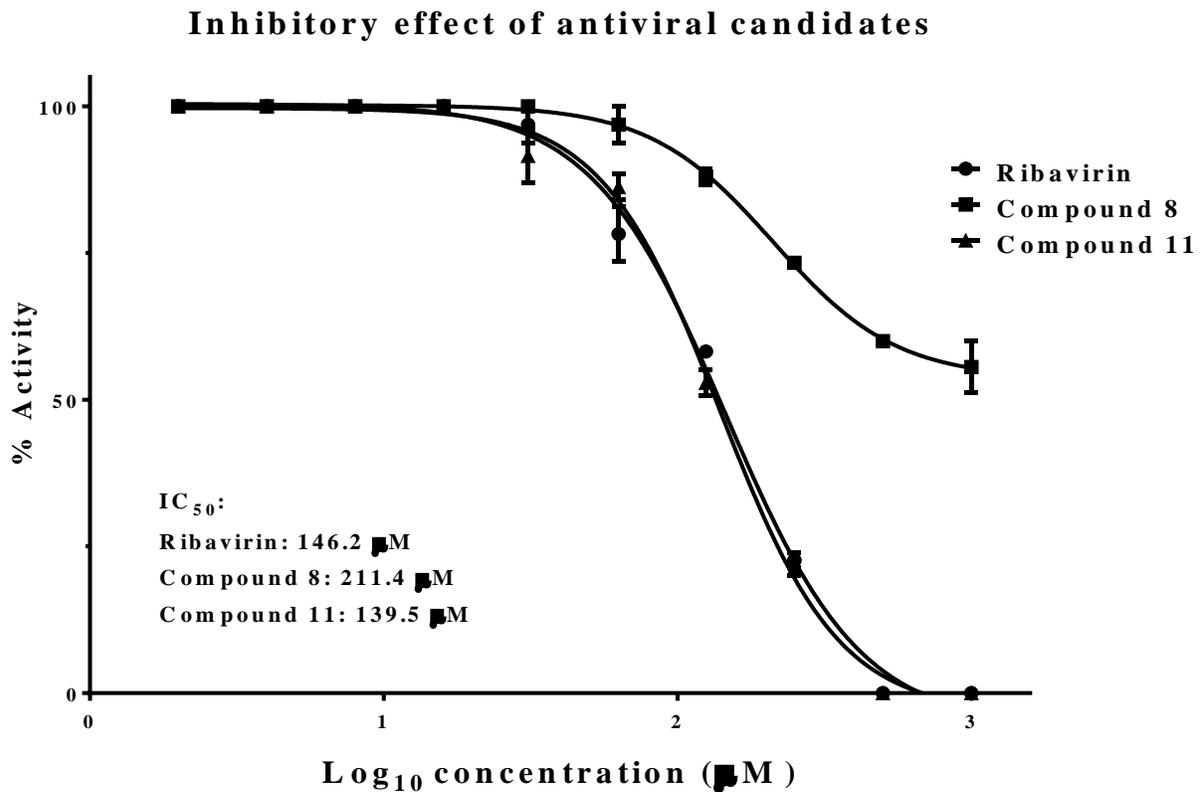


Figure 15 *In vitro* antiviral activity of Compound 8 and 11.

Inhibitory effect of Compound 8, 11, and ribavirin on RVFV MP12 replication in Vero cells. Vero cells were pretreated with serially 2-fold diluted Compound 8, 11, or ribavirin 4 h before infection and were inoculated with RVFV MP12 at an MOI of 2. The virus infectious dose in the culture supernatants and a sigmoidal dose-response curve was fitted to the data using GraphPad Prism 6 (GraphPad Software).

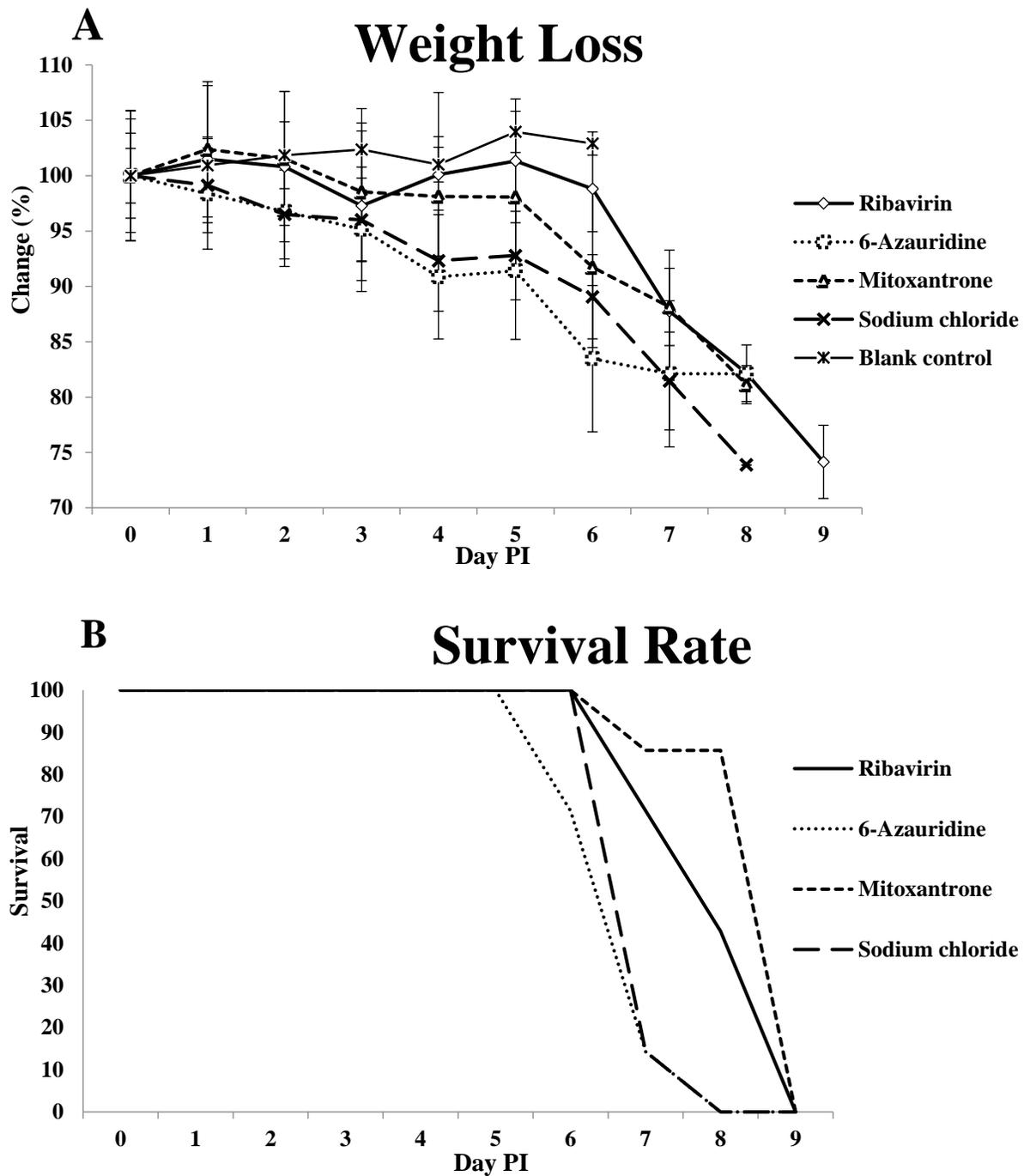


Figure 16 Weight loss and survival rate of mice infected with MP12 and treated with different candidates.

A: Weight of mice in ribavirin group decreased from 1 dpi to 3 dpi and then increased till 5 dpi. After 5 dpi, the weight of mice decreased dramatically. Mitoxantrone treated group showed slower weight loss than the placebo (Sodium chloride) group; B: Mice in ribavirin and mitoxantrone groups survived one more day than the placebo (Sodium chloride) group.

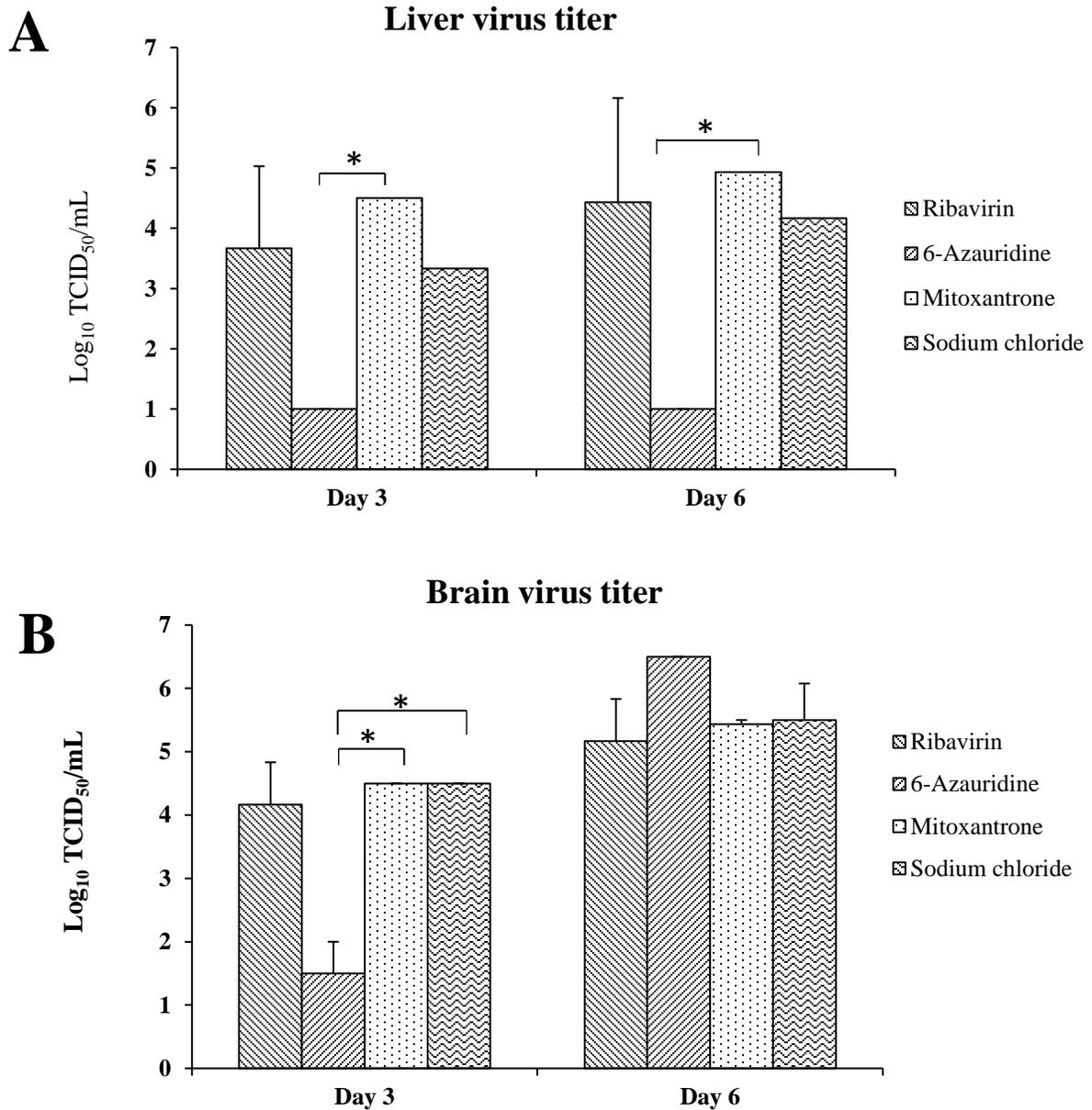


Figure 17 Virus titers in mouse livers and brains collected on 3 and 6 dpi.

A: Virus was detected in mice from all groups on 3 and 6 dpi. A lower virus titer was found in mouse livers of the 6-azauridine group; B: Virus was detected in mice from all groups on 3 and 6 dpi. A significant lower virus titer was found in mouse brains of the 6-azauridine group on 3 dpi. Error bars represent \pm SEM (* $P < 0.05$).

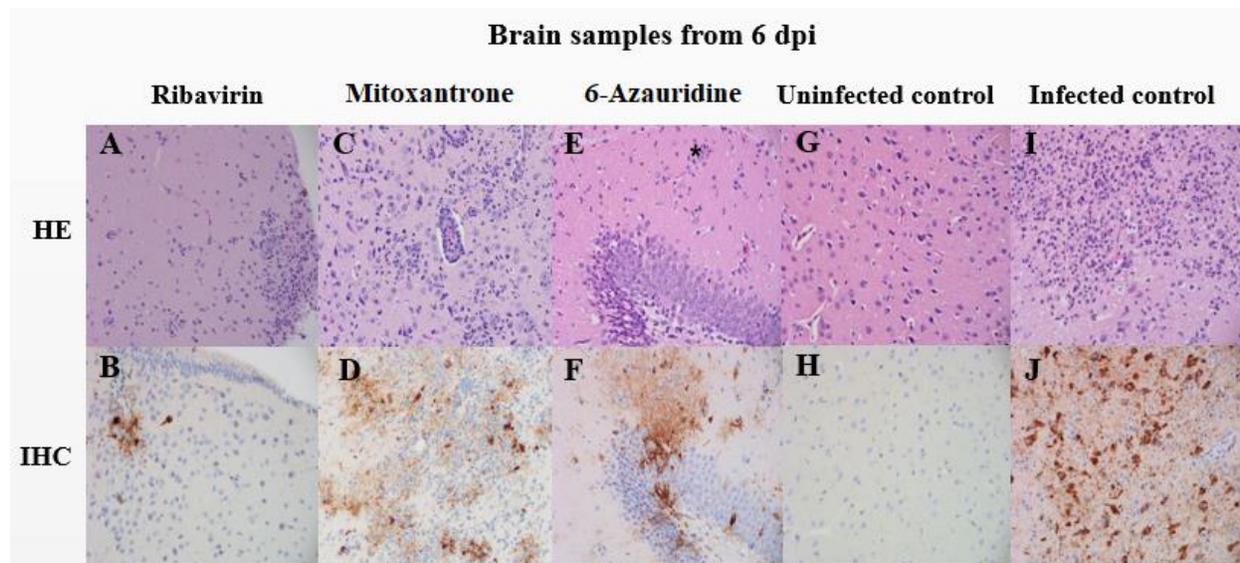


Figure 18 H&E and IHC results from mouse brain samples collected at 6 dpi.

A, C, E, G, and I: H&E results from different groups. A): There is a focal area of necrosis in the mouse from the ribavirin group, the remainder of the brain section is normal. C): In the mouse from the mitoxantrone treatment group, the brain has necrosis with necrotic cellular debris. E): There is a single small focal aggregate of inflammation (*) in the neuropil in this mouse from the 6-azauridine treatment group. G): No lesions consistent with normal brain of a non-challenged control mouse. I): In the mouse from the sodium chloride treatment group, the brain has extensive necrosis with abundant necrotic cellular debris. This mouse also had meningitis, which is not pictured. B, D, F, H, and J: IHC results from different groups. B): A focal area of RSVFV antigen deposition is present in neurons and the neuropil. D): RSVFV antigen is present in the neurons and the neuropil in mitoxantrone 1 treated mouse brain. F): Moderate RSVFV antigen is present in the neurons and the neuropil. H): No RSVFV antigen was present in the brain of a non-challenged control mouse. J): Extensive RSVFV antigen is present in the neurons and the neuropil. (H& E and IHC RSVFV, $\times 400$).

Chapter 4 - General Discussion and Conclusion

Since the 1960s, antivirals have been used to treat certain viral infections (Heymann, Gulick, De Boer, De Stevens, & Mayer, 1958; Hurst & Hull, 1956; Tsuji, 1954). Approximately 90 compounds were formally licensed to use as clinical antiviral therapies, half of them treating human immunodeficient virus (HIV) infection (De Clercq, 2004; De Clercq & Li, 2016). Other approved antiviral treatments act on hepadnavirus (HBV), hepacivirus (HCV), herpes simplex virus (HSV), influenza viruses, human cytomegalovirus (HCMV), varicella-zoster virus (VZV), respiratory syncytial virus (RSV), and human papillomavirus (HPV) (De Clercq & Li, 2016). Although these antivirals are effective against specific viral infections, a few broad-spectrum antiviral drugs are effective against a wide range of viruses (Aman et al., 2009; Caroline et al., 2014; Hoffmann, Kunz, Simon, Palese, & Shaw, 2011; Snell, 2001). Because of specific licensing, antiviral therapies are still unavailable for quite a lot of critical transboundary and emerging viral infections.

Currently, there are no licensed antiviral drugs approved for RVFV treatment in the U.S. As a Category A pathogen, virulent RVFV strains can only be handled in a BSL-3 or higher facility, limiting RVFV antiviral development (D. Bishop & Shope, 1979; Georgiev, 2009). Due to these facts, our first objective is to establish a suitable mouse model that can be used to test antivirals in a BSL-2 environment using BSL-2 approved RVFV strains. In our study, the STAT-1 KO mouse strain has been shown to develop representative disease characterized by acute-onset hepatitis and delayed-onset encephalitis after intranasal MP12 infection (Boshra, Lorenzo, Rodriguez, et al., 2011; Bouloy et al., 2001; Dodd et al., 2014; Lang et al., 2016; Lorenzo et al., 2010). The immune deficient mouse strain, 129Sv IFNAR^{-/-}, are susceptible to MP12 infection, suffering 100% mortality after infection (Lorenzo et al., 2010). Previously, these mice were used

in RVFV vaccine efficacy studies (Boshra, Lorenzo, Rodriguez, et al., 2011; Lorenzo et al., 2010). After vaccination, IFNAR^{-/-} mice generated an immune response and were protected against lethal RVFV MP12 challenge. Unfortunately, no RVFV specific clinical signs were observed in the IFNAR^{-/-} mouse model, indicating, this model should be sufficient to evaluate prognosis but not for prophylaxis. Due to these factors, we hypothesize the STAT-1 KO mouse can be used to evaluate RVFV antivirals in a BSL-2 environment.

In chapter three, we screened two NIH Clinical Collections for identification of effective antiviral candidates, and they were further evaluated *in vitro* and *in vivo*. Previously, ribavirin has been used as antiviral against RVFV. However, this antiviral drug can only be considered as the treatment for complex RVF disease. In this study, we used it as a positive control. Two candidates, 6-azauridine and mitoxantrone, showed inhibition effect on MP12 replication by the plaque reduction assay with negligible cytotoxicity at the active antiviral concentration *in vitro*. While *in vivo* study showed better outcomes in ribavirin and mitoxantrone treated mice but not in mice received 6-azauridine treatment. Mitoxantrone can inhibit Poxviruses by blocking the virus assembly with unclear mechanism *in vitro* (Deng et al., 2007). Similar with RVFV, vaccinia virus also forms protein precursors of structure proteins (Ngo, Mirzakhanyan, Moussatche, & Gershon, 2016). Therefore, mitoxantrone may employ the same mechanism that restrained vaccinia virus package to inhibit RVFV. While 6-azauridine showed inhibition effect *in vitro* and can inhibit virus replication in livers and brains on the early stage of infection. A broad range of viruses can be inhibited by this synthetic triazine analogue of uridine with unknown mechanisms (Crance et al., 2003; Neyts et al., 1996; Rada & Dragúň, 1977). It is interesting to see that the RVFV MP12 replicated fast in brains from 3 dpi to 6 dpi of 6-azauridine treated mice. This fact

implies that MP12 may gain 6-azauridine resistance in the presence of the antiviral treatment. Therefore, the use of antivirals could be a double-edged sword in some cases.

Besides the side effects, antiviral treatments might lead to even worse situations since they could select drug-resistant strains. Ribavirin, a nucleoside analogue, can inhibit a broad spectrum of viruses *in vitro* with pleiotropic mechanisms including inhibition of viral capping enzymes, lethal mutagenesis of viral RNA genomes, and inhibition of viral RNA synthesis (Graci & Cameron, 2006; R. Smith & Kirkpatrick, 1980). Resistance has been identified in poliovirus although a 99.3% loss in infectivity was observed after ribavirin treatment which caused a 9.7-fold increase in mutagenesis. Poliovirus mutant with only one single amino acid change in the viral RNA-dependent RNA polymerase results in resistance (Crotty, Cameron, & Andino, 2001; Pfeiffer & Kirkegaard, 2003). The mutant RNA polymerase has a greater fidelity compared with that of the parental strain. Different from most antibiotics, specific antivirals were designed to target the viral proteins instead of destroying the pathogen (De Clercq, 2007b).

Neuraminidase (NA) inhibitors (NAIs) are in a group of antiviral therapeutics to treat infection caused by influenza viruses. NA is a surface protein of influenza viruses and its primary function is to cleave sialic acids from the cell surface and subsequently release the newly formed virus particles. The NAIs mimic the structure of sialic acids. Therefore they can bind to the active site of NA and then, the newly synthesized virions cannot be released to spread (Von Itzstein et al., 1993). Currently, there are three Food and Drug Administration (FDA) approved NAIs available for human use in the U.S.: oral oseltamivir, inhaled zanamivir, and intravenous peramivir.

However, zanamivir and peramivir can only be prescribed to ≥ 7 years and ≥ 18 years patients respectively (CDC, 2014; Harper et al., 2009). Because of the consistent use of NAIs, several NAI-resistant mutations were identified in different strains at various places (Ferraris & Lina,

2008; Hatakeyama et al., 2007; Horthongkham et al., 2016). Therefore, viruses can gain resistance to a specific antiviral treatment by mutating over time. As influenza virus is a segmented RNA virus, there are potentials that wild influenza strains would obtain the resistance by reassortment which can lead to an even more complicated situation for treatment. Thus, once we have obtained potential therapeutics for RVFV which is also a segmented virus, human clinical trials should be done carefully. Because antiviral resistant mutations could be a potential risk to public health.

As important viral pathogens caused morbidity and mortality in humans, the treatment for RVFV should not be neglected. While there are arbitrarily 13 functional groups available now for the nine human infectious diseases (De Clercq & Li, 2016). They are: (i) 5-substituted 2'-deoxyuridine analogues (treatment for HSV (De Winter & Herdewijn, 1996), and VZV (McGuigan et al., 1999)); (ii) nucleoside analogues (for HSV (Fyfe, Keller, Furman, Miller, & Elion, 1978), HBV (van Bömmel et al., 2010), and VZV (Balzarini & McGuigan, 2002)); (iii) (nonnucleoside) pyrophosphate analogues (for HCMV (Gilbert & Boivin, 2005), and HSV (Eriksson, Öberg, & Wahren, 1982)); (iv) nucleoside reverse transcriptase inhibitors (for HIV (Group, 2008), and HBV (Margeridon-Thermet et al., 2009)); (v) nonnucleoside reverse transcriptase inhibitors (for HIV (Merluzzi, Hargrave, Labadia, Grozinger, & Skoog, 1990)); (vi) protease inhibitors (for HIV (Condra, Schleif, Blahy, & Gabryelski, 1995), and HCV (C Lin et al., 2004)); (vii) integrase inhibitors (for HIV (Pommier, Johnson, & Marchand, 2005)); (viii) entry inhibitors (for HIV (Reeves et al., 2002), HSV (Gong et al., 2002), VZV (Zhu, Gershon, Ambron, Gabel, & Gershon, 1995), and RSV (Razinkov, Huntley, Ellestad, & Krishnamurthy, 2002)); (ix) acyclic guanosine analogues (for HCMV (Wahren, Larsson, Rudén, Sundqvist, & Sølver, 1987), HSV (De Clercq et al., 2001), and VZV (Karlström, Källander, Abele, & Larsson,

1986)); (x) acyclic nucleoside phosphonate (ANP) analogues (for HIV, HCMV, and HBV (De Clercq, 2007a),); (xi) HCV NS5A and NS5B inhibitors; (xii) influenza virus inhibitors; and (xiii) immunostimulators, interferons, oligonucleotides, and antimetabolic inhibitors (for HBV (Sokal et al., 1998), HCV (Manns et al., 2001), HCMV (Chapman, Thayer, Vincent, & Haigwood, 1991), and HPV (Jach, Basta, & Szczudrawa, 2003)). Unlike the viruses with available treatment, the underlying mechanisms of RVFV entry, replication, and release are poorly understood.

Therefore, screening work from the available antiviral candidates is in great need.

To avoid the generation of directed mutations caused by a single type of treatments, a combination of antiviral therapeutics with different functions should be employed to treat the viral diseases. As more than nine million life-years were saved from HIV infection by combination antiretroviral therapy, we should learn the therapeutic method to control other important pathogens such as RVFV (Joint United Nations Programme on HIV/AIDS, 2012). Last but not least, because encephalopathy was observed in the severe cases of RVFV combination of infections, different formations of treatment should be considered to increase and stabilize the antiviral concentration in the brain (CH Lin et al., 2006; M Pepin et al., 2010; Sazgar, Robinson, Chan, & Sinclair, 2003). As different antivirals have different characteristics such as molecular size, electric charge, lipophilicity, plasma protein binding, and the host factors, it is difficult to predict whether they can penetrate the BBB or not if we treat the host intraperitoneally or intravenously (Nau et al., 2010). Intranasal delivery is a practical method to bypass the BBB to deliver small therapeutic molecules to the brain and spinal cord (Hanson & Frey, 2008). These therapeutic agents can be delivered to the brain by olfactory and trigeminal pathways which providing a direct connection with the CNS (Dixon et al., 2016; Ross et al., 2004; Thorne et al., 2004). In addition, Colombo et al. reported that ribavirin could transport from the nose to brain

rapidly after nasal administration in the powder formation (Colombo et al., 2011), indicating intranasal route could be an alternative way to administrate antiviral treatment for RVFV infection.

Taken together, our studies cast light on the antiviral development against RVFV *in vitro* and *in vivo*. To provide a better therapeutic method for this important viral disease lacking attentions, we will identify and develop more effective antiviral drugs against RVFV with different antiviral mechanisms. Meanwhile, antiviral mechanisms of 6-azauridine and mitoxantrone against RVFV infection will be further studied. In addition, a combination therapy will be developed with RVFV antiviral candidates in the future as well.

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