The development of novel diagnostic countermeasures for Rift Valley fever virus

by

Izabela Katarzyna Ragan

B.A., The Colorado College, 2009
D.V.M., Kansas State University, 2014

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2018

Abstract

Rift Valley fever virus (RVFV) is a zoonotic arbovirus that is a significant threat to livestock and humans. It is listed as #3 for most dangerous animal threats and is in the top 10 pathogens needing urgent research in preventative and control measures. Although RVFV has never been reported in the US or Europe, outbreaks outside the African continent have sparked renewed interest in developing diagnostics and vaccines to protect both agriculture and public health. Having specific and versatile diagnostics is critical for vaccine development and application. For example, diagnostic tools that aid in identifying key immunogens and understanding the virus-host interaction directly contribute to developing protective vaccines. Additionally, vaccines that are used prophylactically or in response to an outbreak require diagnostic tests to differentiate infected from vaccinated animals (DIVA). This is critical for assessing the return to 'disease free' status after an outbreak. Unfortunately, there are limited RVFV diagnostic tests that are versatile and DIVA compatible with the newest RVFV vaccines. We describe the development of several diagnostic tools that are DIVA compatible for detecting RVFV nucleic acid, antibodies, and antigens. First, we evaluate a fluorescence microsphere immunoassay (FMIA) for the detection of antibodies against a RVFV surface glycoprotein and the nucleocapsid protein. The targets developed in this assay provide the basis for a DIVA-compatible serological assay with a candidate RVFV Gn/Gc subunit vaccine, as well as, offer a multiplexing platform that can simultaneously screen for several ruminant diseases. Second, we describe a novel chromogenic in situ hybridization (ISH) assay to detect RVFV in formalin-fixed, paraffin-embedded (FFPE) tissues. This molecular assay offers a highly sensitive, multiplexing platform that detects RVFV RNA on the cellular level of diagnostic tissue samples. Moreover, we demonstrate the first application of ISH as a DIVA-compatible assay for candidate

RVFV gene-deletion vaccines. Third, we provide working protocols for western blot (WB), immunohistochemistry (IHC), and immunofluorescence (IF) that use monoclonal or polyclonal antibodies against key RVFV antigens. These tools can be applied to pathogenesis research and used in the development of vaccine and therapeutic countermeasures against RVFV. The RVFV diagnostic methods developed and evaluated in this dissertation can serve as a model for developing diagnostic strategies for other transboundary animal diseases.

The development of novel diagnostic countermeasures for Rift Valley fever virus

by

Izabela Katarzyna Ragan

B.A., The Colorado College, 2009
D.V.M., Kansas State University, 2014

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2018

Approved by:

Co-Major Professor
Dr. A Sally Davis

Approved by:

Co-Major Professor
Dr. William C. Wilson

Copyright

Abstract

Rift Valley fever virus (RVFV) is a zoonotic arbovirus that is a significant threat to livestock and humans. It is listed as #3 for most dangerous animal threats and is in the top 10 pathogens needing urgent research in preventative and control measures. Although RVFV has never been reported in the US or Europe, outbreaks outside the African continent have sparked renewed interest in developing diagnostics and vaccines to protect both agriculture and public health. Having specific and versatile diagnostics is critical for vaccine development and application. For example, diagnostic tools that aid in identifying key immunogens and understanding the virus-host interaction directly contribute to developing protective vaccines. Additionally, vaccines that are used prophylactically or in response to an outbreak require diagnostic tests to differentiate infected from vaccinated animals (DIVA). This is critical for assessing the return to 'disease free' status after an outbreak. Unfortunately, there are limited RVFV diagnostic tests that are versatile and DIVA compatible with the newest RVFV vaccines. We describe the development of several diagnostic tools that are DIVA compatible for detecting RVFV nucleic acid, antibodies, and antigens. First, we evaluate a fluorescence microsphere immunoassay (FMIA) for the detection of antibodies against a RVFV surface glycoprotein and the nucleocapsid protein. The targets developed in this assay provide the basis for a DIVA-compatible serological assay with a candidate RVFV Gn/Gc subunit vaccine, as well as, offer a multiplexing platform that can simultaneously screen for several ruminant diseases. Second, we describe a novel chromogenic in situ hybridization (ISH) assay to detect RVFV in formalin-fixed, paraffin-embedded (FFPE) tissues. This molecular assay offers a highly sensitive, multiplexing platform that detects RVFV RNA on the cellular level of diagnostic tissue samples. Moreover, we demonstrate the first application of ISH as a DIVA-compatible assay for candidate

RVFV gene-deletion vaccines. Third, we provide working protocols for western blot (WB), immunohistochemistry (IHC), and immunofluorescence (IF) that use monoclonal or polyclonal antibodies against key RVFV antigens. These tools can be applied to pathogenesis research and used in the development of vaccine and therapeutic countermeasures against RVFV. The RVFV diagnostic methods developed and evaluated in this dissertation can serve as a model for developing diagnostic strategies for other transboundary animal diseases.

Table of Contents

List of Figures

List of Tables

Acknowledgements

First of all, I would like to thank my co-advisors, Dr. Sally Davis and Dr. William Wilson. I thank Dr. Davis for allowing me to explore my interests and pushing me to excel in my work. And I thank Dr. Wilson for the continuous guidance and patience through the entirety of my PhD. I am honored to have worked with such amazing mentors.

I would like to express my sincere gratitude to my committee for guiding me through my PhD training. Thank you to Dr. Stephen Higgs, Dr. Elizabeth Davis, Dr. Scott McVey, and Dr. Bob Rowland. It has been a privilege working with such a supportive committee. I appreciate all the ideas and the encouraging words.

I thank KSU Center of Excellence for Emerging and Zoonotic Animal Diseases for supporting my fellowship and allowing me to expand my training in biocontainment research.

My sincere thanks to all my lab mates and colleagues. Having the opportunity to work in several labs I am blessed to have so many friends that have supported me through thick and thin. Special thanks to Dane Jasperson, Erin Schirtzinger, Lindsey Reister, Barbara Drolet, Jessie Trujillo, Nick Monday, Vlad Petrovan, Johanna Lindahl, Deepa Upreti, Maryka Smith, Monica Gamez, Mal Hoover, Peying Fong, Ross Wahl, Rachel Palinski, Mollie Burton, Mallory Heimerman, and Kathleen Yeater who helped me put this dissertation together. Your generous help will never be forgotten.

Most importantly, I thank my husband, Mario Martinez. Despite living in separate states during this PhD, he was still my biggest cheerleader. Without his love and devotion, I would not be here. No words can describe my gratitude for his staying by my side while I pursued my dreams.

Dedication

"I will praise the LORD, who counsels me; even at night my heart instructs me.
I keep my eyes always on the LORD. With him at my right hand, I will not be shaken.
Therefore my heart is glad and my tongue rejoices; my body also will rest secure,
because you will not abandon me to the realm of the dead, nor will you let your faithful one see decay.
You make known to me the path of life; you will fill me with joy in your presence, with eternal pleasures at your right hand."

~Psalm 16:7-11 (NIV)

Chapter 1 - Literature Review

Introduction

Rift Valley fever virus (RVFV) is a zoonotic, mosquito-borne pathogen of great economic and public health importance (1). It is a reportable disease under the World Organization for Animal Health (OIE) Terrestrial Animal Health Code (2). With the re-emergence of viruses like Zika in 2016 and Ebola in 2014, RVFV and other transboundary animal pathogens are on the radar as a threat to human health (3). Factors such as climate changes that are expanding mosquito distribution, growing agricultural trade, and an increase in bioterrorism threats have made Rift Valley fever (RVF) disease a critical international priority. Recently reported African outbreaks of RVFV have led to a renewed urgency for innovative diagnostics and vaccines to combat this disease. It is important to focus on pathogen control as a global problem and develop diagnostics applicable to both endemic and non-endemic countries. Therefore, an emphasis should be put on universal and versatile diagnostic platforms to support coordinated global containment efforts. Successful control of RVFV would pave the way for the management of other transboundary animal diseases.

The virus

RVFV is classified under the order *Bunyavirales*, family *Phenuiviridae*. The virus is an enveloped spherical virus approximately 120 nm in diameter. The genome is tripartite consisting of small (S), medium (M), and large (L) segments (Figure 1.1) (4). All three segments are packaged with viral nucleocapsid (N) protein and the viral RNA-dependent RNA polymerase to form ribonucleoprotein complexes (RNP). The RNP within the inner core is separated by a lipid bilayer. The viral envelope contains glycoprotein projections of 10 nm in diameter that are

anchored in an icosahedral lattice. Unlike other bunyaviruses, RVFV does not have a matrix protein (5). Understanding the structural organization of RVFV aids in the development of diagnostic assays that target important viral components and epitopes.

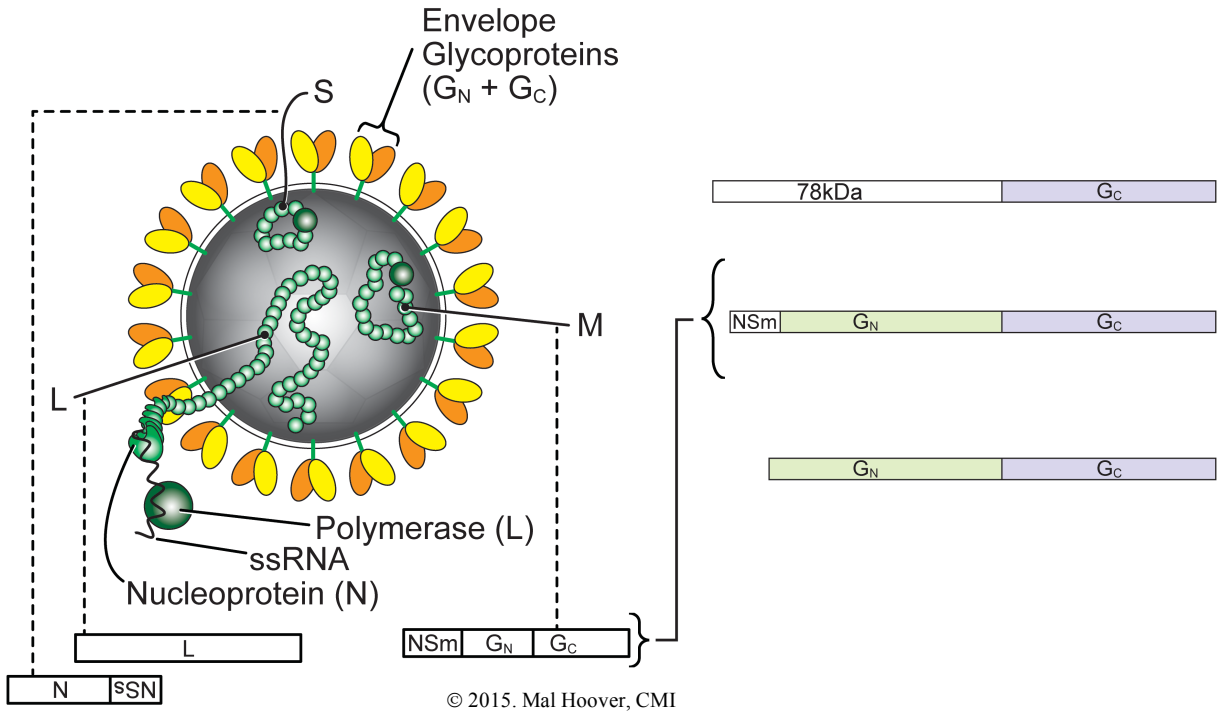

Figure 1.1 Structure of RVFV virion

The S segment (1690 nucleotides in length) is an ambisense segment that encodes the nucleocapsid protein (N) and the nonstructural protein (NSs). The N protein is transcribed in the viral-sense orientation while NSs is transcribed in the viral-antisense orientation (6). With the L polymerase, the N protein forms RNPs that associate with viral RNA and cDNA to assist in viral transcription and replication (7). The N protein is the most abundant protein in the virion and is considered to be highly immunogenic, making this protein an ideal target for diagnostic development (8, 9). The NSs protein is a major virulence factor. It forms nuclear filaments that disrupt host nuclear proteins and inhibiting transcription (10, 11). It also promotes apoptosis as

well as blocks interferon (IFN-β) and mediates early Protein Kinase R degradation to counter cellular antiviral activities (12-15). Therefore, NSs is an important target for attenuating the virus (16, 17).

The M segment (3885 nucleotides in length) encodes for two glycoproteins (Gn, Gc) and two minor proteins, a 78-kDa nonstructural protein and the 14-kDa nonstructural protein (NSm) (18). Depending on where the initiation of translation occurs, cleavage of the M polyprotein can yield the two glycoproteins, Gn and Gc, the two glycoproteins with NSm, or Gc with the 78-kDa nonstructural protein. NSm is believed to suppress virus-induced apoptosis and contributes to establishing host infection (19). This comes from the observation that RVFV recombinant strains lacking the 14-kDa NSm expression are attenuated (20), although they can still cause clinical disease (21). The larger 78-kDa protein may be important in virus dissemination in mosquitos (20, 22). Gn (54-kDa) is thought to be involved in receptor binding while Gc (56-kDa) plays a major role in membrane fusion (23). The glycoproteins elicit the production of neutralizing antibodies and are a correlate of protective immunity (24). Therefore, the glycoproteins have become widely used as targets in serological assays and vaccines to monitor immune protection against RVF infections.

The L segment (6404 nucleotides in length) encodes for the RNA-dependent RNA polymerase that with N, is required for viral RNA synthesis (25). Like other bunyaviruses, RVFV uses a ‘cap-snatching’ method for mRNA transcription (26). Because of the large size of the segment and its highly conserved nucleic acid sequence among RVFV strains, the L segment is a good target for molecular diagnostic development.

Transmission

RVFV poses a significant global threat because it can be transmitted by multiple routes. Mosquitoes are an important vector for transmission to animals and are essential in maintaining an enzootic cycle. Whereas direct contact with infected tissues and bodily fluids is a major risk factor for transmission to humans (27). Humans can also acquire the virus by mosquito bites, vertical transmission (28), infected milk (29), and aerosolization (30) although less frequently when compared to direct contact. The zoonotic potential of RVFV has resulted in its designation as an Overlap Select Agent (31). Currently, there is no licensed human vaccine for this disease. Therefore, rapid and accurate diagnosis is critical during an outbreak to minimize the spread of RVFV.

With several modes of transmission, the risk of RVFV spreading outside of its endemic areas is high. The first likely scenario would be by wind-borne spread of infected mosquitoes as seen in the 1977 outbreak when RVFV spread from Sudan to Egypt. Another likely route would be by transportation of viremic animals and/or people to non-endemic countries with competent vectors, which was observed in the 2000 Saudi Arabian outbreak (32). Due to the challenges of vector control and increasing international livestock trade, we must rely on accurate diagnostic tests for early detection in outbreaks and for surveillance.

Epidemiology

The virus was first isolated in 1930 from sheep in a forest in Uganda after reports of aborting ewes and high lamb mortality (33). Periodic outbreaks in African countries have been reported along the east and south coasts of Africa and in Madagascar since then (34). The disease is now considered to be endemic in sub-Sahara Africa and outbreaks are still being reported

during episodes of heavy rainfall and flooding as seen in Niger (2016), and Mauritania (2012-2016) (35).

RVFV can move outside traditionally endemic areas by the movement of viremic livestock to area with a high vector density. This poses a huge risk for human infections in ranchers and others handling the livestock (36). The first report of RVFV spreading outside the African continent occurred in 2000 when an outbreak occurred in the Middle East. It is believed that the introduction of RVFV occurred during the transport of infected livestock from East Africa to the Arabian Peninsula (37). With the potential for movements of viremic animals between countries there is concern that RVFV could spread to Europe and cause public health issues (38). International cooperation and monitoring is critical in controlling the disease.

Other factors that facilitate the spread of RVFV include agricultural development and climate change leading to changes in rain patterns and an increase in vectors (39-41). Most outbreaks in Eastern and Southern Africa are linked to heavy rain fall and flooding while those in Northern and Western Africa are associated with irrigation practices (34). In both situations, there is a dramatic increase in RVFV-competent mosquitoes and viral dissemination. Several RVFV control measures have been discussed and implemented including tighter regulation of livestock movement, vector control, vaccination, and public education (42). No single measure can be successful independently. Instead these measures need to be implemented together with detection tools and global support to stop the spread of RVFV.

The Threat of RVFV

Economic threat

The economic impact from the introduction of RVFV into Europe and/or the U.S. would be devastating. As with many emerging diseases, including SARS in 2003 and Ebola in 2014, there would be major socioeconomic impacts. Tourism, healthcare and business sectors can be impacted globally (1, 43). Because of its zoonotic potential, RVFV may create public panic due to fear of contracting the disease through contact with infected animals and/or tissues. Ruminants are highly susceptible to infection leading to high morbidity in adults and high mortality in newborns. As seen with foot-and-mouth disease (FMD), highly contagious viral pathogens can lead to high economic costs due to animal testing and quarantine, trade embargos, and destruction of infected animals. A few reports from African countries that have estimated substantial economic losses from RVFV including US $32 million in Kenya during the 2007 outbreak (44) and over US $75 million per year in Saudi Arabia (45). The magnitude of economic loss that would be expected if an outbreak occurred in the US is still unclear. Immediate costs would include mitigation costs and loss of animals leading to a disruption in the food supply chain. Longer term costs would come from potential vaccine production and administration as well as international trade restrictions (46). Early detection of zoonotic pathogens is critical for protecting agriculture and public health (47). Therefore, investment in the development of early-detection tests for RVFV in livestock will reduce the economic impact of this transboundary animal pathogen in non-endemic countries.

Bioterrorism

RVFV has the potential to be used as a bioterrorism weapon because of its high morbidity and mortality rates, its ability for zoonotic transmission, and the potential for large-

scale production (48). Another key factor is its potential for aerosol dissemination allowing for easy dispersion over large populations. RVFV is very stable in the environment and believed to require a low aerosol dose to cause infection (49). Because of these properties, the virus is currently listed as a Category A agent by the CDC and the USDA. RVFV is considered a high priority transboundary disease with devastating international impacts.

The need to monitor and control

RVFV is a significant viral threat to both endemic and non-endemic countries, therefore, it is important to monitor the disease. Inadequate surveillance in animals and humans can lead to delayed detection and response. In turn, the severity of RVFV outbreaks may increase (50). Meteorological forecasting of heavy rainfall in endemic countries has been shown to be a valuable tool in predicting RVFV emergence. In 2006, mathematical modeling was used to produce a risk map that successfully predicted an outbreak in the Horn of Africa (51). Despite having a 2-6 week warning period in which a response and surveillance program were implemented, human cases were reported across multiple countries. Future research needs to focus on rapid vaccination and diagnostic tests to improve preventative measures and limit the spread of the disease. Because RVFV emerges in irregular cycles followed by long inter-epizootic periods, efforts should be focused on monitoring enzootic activity in livestock (34). Livestock can reach high levels of viremia leading to transmission to mosquitoes and humans. Furthermore, livestock have the potential to establish RVFV in non-endemic areas. Therefore, monitoring should include regular testing of aborted fetuses and serological testing of livestock operations near water sources.

RVF Disease

RVFV causes disease in both animals and humans leading to devastating losses. The virus has broad tissue and cell tropism. It replicates primarily in the liver, lymphoid tissue such as the spleen, and in some cases the brain. Ikegami, et al., 2011 provides a summary of RVFV pathology in humans and various animal species (52). Ross, et al., 2011 describes the pathobiology of key animal models used to study RVFV (53). Finally, Odendaal, et al., unpublished provides an in-depth evaluation of RVFV pathology in naturally infected adult sheep.

Disease in animals

RVFV can infect several animal species including ruminants, camelids, rodents, non-human primates, and household pets (33, 54-57). Animals from the families *Bovidae, Camelidae,* and *Muridae* as well as non-human primates are used as the primary animal models for RVFV studies. Conversely, clinical disease is not apparent in guinea pigs, bats, rabbits, horses, pigs, reptiles, and birds (58-60). RVFV infection causes limited morbidity and mortality in African wildlife but they may serve as epidemiological reservoirs. Antibodies have been detected in African wildlife including *Rhinocerotidae, Cervidae, Bovidae,* and *Elephantidae* families (60-62). Yet, little is known about wildlife hosts in non-endemic countries. Cell lines from livestock, wild ruminants, pigs, frogs and coyotes are permissive and susceptible to RVFV replication (63). Additionally, there is ongoing research into the potential role of U.S. white-tailed deer as an animal reservoir for RVFV (Wilson et al., unpublished). Nonetheless, more research is needed to identify competent wildlife hosts and potential ecological reservoirs for RVFV in the US and Europe. This knowledge would be extremely valuable for predicting the economic impact and zoonotic risk in the event of an RVFV introduction.

Bovidae

Domestic livestock, including sheep, goats, and cattle, are important primary hosts for RVFV. Abortion storms, fetal malformation, and high neonatal mortality are common in sheep and cattle during outbreaks. Sheep are considered to be the most susceptible to RVFV infections. Since the first reported outbreak on a sheep farm in 1930, RVF has been described in various breeds as well as in both young and adult animals (33). Newborn lambs infected with RVFV develop acute disease and have a mortality rate near 95% (64). The RVFV incubation period in lambs is about 12 to 36 hours (hr) after which they become weak, febrile and show signs of abdominal pain. Death can occur 12 hr after the onset of pyrexia. Older animals are less susceptible and have variable clinical signs. The incubation period is slightly extended in adults to 24-72 hr and is followed by gastrointestinal signs, lymphadenopathy, icterus, and pyrexia. Unlike the high mortality seen in lambs, the adult mortality is lower ranging from 10 to 70% (34). Pregnant ewes are at a high risk of abortion and death with a mortality rate of about 20% (65). The divergence of host genetics affects the susceptibility and clinical signs among various breeds (52). Because of their susceptibility, sheep are important animal models in vaccine development and pathogenesis research.

Goats are generally less susceptible to RVF and have lower abortion and morbidity rates than sheep (38). Their clinical signs can vary depending on the route of infection and may include lethargy, mild fever, and diarrhea. A recent experimental challenge study showed that goats are less viremic for a shorter amount of time than sheep (66). Yet, goats are still suitable models for RVF studies in small ruminants and have been used to study the innate immune response to RVFV (67).

Cattle are susceptible to the disease but to a lesser extent than sheep. Although, they are considered to be more susceptible than goats. Juvenile calves have a mortality rate close to 40%, while adult mortality is only approximately 10%. Calves can have a peracute infection and die within 24 hr. Clinical signs of experimentally infected adult cattle include inappetence, dullness, dysgalactia, icterus, and blood-stained, mucoid nasal discharge, and abortion (34). Alternatively, cows may be asymptomatic. The aborted fetus is usually autolyzed, making testing and diagnosis potentially difficult (34, 68). As with sheep, cattle are used frequently for experiential challenge and vaccine efficacy studies.

Camelidae

Camels can become infected with RVFV which can lead to abortion storms (56). Like cattle, camels can be asymptomatic when infected (34). Camels that do show clinical signs have an acute infection with nasal discharge, oral hemorrhage, foot lesions, icterus, conjunctivitis, fever, and abortion (69). A peracute form of the disease can lead to sudden death within 24 hr (70). Several African countries have reported seroprevalance of RVFV in camels (69). Because of this, it is important to include these animals in disease surveillance and evaluate the zoonotic potential. Camels are not commonly used as animal models for RVFV research due to their size. Instead, alpacas can serve as a model for RVFV infection in dromedary camels as already reported in an MP12 vaccine efficacy study (71).

Muridae

Rodents are highly susceptible to RVFV and therefore are a cost-effective animal model. Mice, Syrian hamsters, rats, and gerbils have been used to examine the hepatic and/or neurologic disease caused by RVFV.

Mice are highly susceptible to RVFV infections. They develop severe hepatitis and die as early as 3 days post infection (dpi). Those that recover from hepatic disease may succumb to encephalitis around 8 dpi (72). Mice have been used to examine how the route of RVFV inoculation influences clinical outcome. For example, intranasal or aerosol exposure leads to neuropathology (30, 73). Furthermore, inoculation of mice by intraperitoneal injection leads to replication of the virus primarily in the liver, while intradermal inoculation leads to both liver and neurological disease (74). Mouse models are in wide use for virulent RVFV infections because of this disease profile.

Syrian hamsters are the most susceptible of all rodents to RVFV (33). Because they develop acute, fulminating hepatitis without the progression to neurological disease hamsters are more advantageous for testing RVFV therapeutics (75).

Rats are moderately susceptible to RVFV infection. Rats demonstrate RVFV-associated hepatitis and encephalitis and also an age dependent susceptibility where less than 2 week old rats die in 2-4 days while 26 day old rats can survive RVFV infection (76). Additionally, there are differences in susceptibility among various inbred rat strains (57). Therefore, rats are used for investigating host genetics' influence on RVFV susceptibility.

Susceptibility of gerbils is also considered to be moderate. They differ from other rodent models in that in them RVFV causes neurological disease with minimal liver involvement. Resistance to encephalitis is age dependent with younger animals having a higher mortality rate. Interestingly, the development of encephalitis is viral dose independent (77). Therefore, gerbils have become an important animal model for studying RVFV-induced encephalitis.

Non-human primates

Like humans, non-human primates are considered to be moderately susceptible to RVFV. Hemorrhagic fever-like disease has been described in Rhesus macaques but only a small percentage of infected animals develop these signs (78). One study presents marmosets as an ideal animal model because their disease profile is most similar to that seen in severe human RVFV infections (79). The incubation period in the marmoset is about 2-4 days and is followed by weakness, vomiting, anorexia, petechial hemorrhages, and depression. A coagulopathy may occur leading to disseminated intravascular coagulation (80). It appears that South American monkeys are more susceptible and become febrile from experimental inoculation of RVFV compared to African monkeys (81). Non-human primates are valued animal models for the development of vaccines and therapeutics for humans.

Disease in humans

Human infections can lead to a wide range of clinical signs, but the infection is usually self-limiting with mild, flu-like symptoms. The incubation period is about 2-6 days in humans. Clinical cases can lead to severe disease characterized by biphasic fever, encephalitis, hepatitis, retinitis, and/or a hemorrhagic syndrome (82). The disease can progress to hepatic and renal dysfunction as well as disseminated intravascular coagulation (83). Reports from the 2007-2008 Sudan RVFV outbreak revealed that severe clinical cases can also have acute renal damage (84). The fatality rate is about 2% (85). Identification of RVF by clinical signs typically occurs later during an outbreak because of its non-specific symptoms and low incidence of the hemorrhagic syndrome. Yet, understanding the clinical symptoms in humans and the ability to identify RVF cases early is critical to mitigating future human RVF casualties (50).

Vaccines

The vaccination of livestock supports long term strategies in minimizing the impact on agriculture from RVFV infections (86). Two important vaccine strategies against RVFV currently being evaluated are live attenuated and subunit vaccines. Additional strategies include inactivated, vector-based, DNA-based, and virus-like particles (VLP)-based vaccines. An ideal RVFV vaccine should: confer protection after a single immunization, provide safe production outside biocontainment, not revert to virulence, maintain stability for long term storage, and be low cost. More importantly, RVFV vaccines need to contain markers that will enable the differentiation of infected from the vaccinated animals (DIVA). DIVA-compatible vaccines help in disease surveillance and enable countries to use vaccination as a control method while maintaining "disease-free" status. Currently, several live attenuated and subunit vaccine platforms are being evaluated as DIVA-compatible vaccines for RVF. These candidate vaccines will require diagnostic assays that can detect their DIVA markers. Both vaccines and companion diagnostic tests are critical to implementing surveillance and control measures for RVFV.

Live attenuated vaccines

Live attenuated vaccines are favored for their ability to rapidly induce protective immunity without requiring additional boosters. The RVFV live-attenuated vaccines include Smithburn, Clone 13, MP12, and several reverse genetics-derived vaccines.

The Smithburn vaccine, a RVFV strain isolated from mosquitoes in Uganda and serially passaged in mouse brains, is one of the oldest vaccines developed to control RVF in Africa (87). The vaccine is low cost and induces long-lasting immunity, which is ideal in endemic countries. Despite the extensive passaging and consequent attenuation of this strain, there is still a potential for its reversion to virulence (88). Additionally, the vaccine has been shown to cause abortions in

livestock (89). The Smithburn vaccine is not suitable for non-endemic countries but may be valuable to nomadic farmers in Africa where higher costs and the need for booster vaccination may prohibit the use of newer and safer vaccine alternatives.

Clone 13 vaccine has a natural large deletion of the NSs segment leading to its attenuation. It is derived from a plaque isolate from a human infected with RVFV in Central Africa (90). The vaccine does not cause any clinical signs of RVFV and is highly immunogenic in sheep and cattle (91, 92). However, a recent study showed that Clone 13 can cross the sheep placental barrier causing fetal infections and malformations (93). The vaccine is licensed in Kenya, South Africa, Namibia, and Botswana (94). Because of the large deletion in its NSs gene, there is an interest in developing DIVA diagnostics tests to detect NSs.

MP12 is an attenuated vaccine derived from the Egyptian strain ZH548 after 12 serial passages in the presence of mutagen 5-fluorouracil resulting in point mutations in all genomic segments. The vaccine was shown to be immunogenic and safe for ewes and younger lambs (95). However, a later study in South Africa showed that MP12 can cause abortions and teratogenesis in sheep (96). Additionally, experimental inoculation of 4-month-old calves with MP12 caused multifocal hepatic necrosis positive for RVF viral RNA (97). While MP12 is genetically stable at low passage on cultured cells, questions regarding its stability when serially passaged more extensively and the virulence associated with generated mutant genotypes remains (98). The vaccine was tested for human use in a phase II clinical trial and there was a 95% antibody seroconversion rate (85). Currently, the vaccine is conditionally licensed for animals in the U.S. (99).

Reverse genetic systems have been used to create RVFV deletion mutants and improve the safety of live attenuated vaccines. Recent work has focused on deletions in the NSm and/or

NSs genes. Two major RVFV strains have been evaluated for their efficacy after gene deletion; ZH501 (16, 17, 21) and MP12 (17, 100-104). More recently, RVFV strain 35/74 was used as a backbone strain to develop a four-segmented variant where the M segment was divided in two (105). Such large deletions and genetic rearrangements can lead to a highly attenuated RVFV strains and are DIVA compatible.

Subunit vaccines

Subunit vaccines have become popular in the last few years because they can be produced outside of high containment. Additionally, subunit vaccines do not require harsh inactivating agents that could alter important viral epitopes. Several subunit vaccines based on the recombinantly produced glycoproteins, Gn and Gc, (106-108) or the N protein (107, 109) have been investigated. Vaccination with the N protein conferred only partial protection in mice due the production of non-neutralizing antibodies for antibody-dependent cell-mediated cytotoxicity (109). Conversely, vaccination with the glycoproteins conferred a high-level of protection by inducing a strong neutralizing antibody response. A candidate vaccine with both Gn and Gc induced the production of neutralizing antibodies in sheep within 2 weeks after primary vaccination (106). The detection of antibodies against immunogenic RVFV antigens such as N and the glycoproteins enables subunit vaccines to be DIVA compatible.

Other vaccine strategies

Inactivated vaccines

Some of the first RVFV vaccines developed were formalin-inactivated. These vaccines, which include NDBR103 and TSI-GSD-200 derived from the Entebbe RVFV strain, give long-term immunity (110, 111). The TSI-GSD-200 vaccine has been evaluated as a candidate vaccine for human use (110, 112). Nonetheless, the vaccines require several primary doses and therefore

are not effective for use in rural communities (113). Also, the vaccines are considered to be less immunogenic than live-attenuated vaccines although they are safer by eliminating the potential for reversion (114).

Vector vaccines

Viral vectors have become a promising vaccine platform to deliver viral genes of interest and support antigen expression intracellularly. In turn, they can stimulate both the humoral and more importantly the cell-mediated immune response to induce long-term immunity (115). Several vector formats have been explored to express RVFV antigens. These includes Newcastle disease virus (108), poxvirus (107, 116-118), alphavirus (119, 120), adenovirus (121-123), and equine-herpes virus type 1 (124). These vaccines still need additional evaluation for use in veterinary medicine.

DNA and virus-like particle vaccines

DNA-based vaccines have been evaluated as a low-cost and temperature-stable option but require multiple priming doses to induce a strong immune response (125). RVFV DNA encoding for the glycoproteins or the nucleocapsid have been evaluated (107, 126, 127). RVFV DNA-based vaccines have also been tested in conjunction with DNA from other viruses as a cocktail vaccine (128). Because of their strong immunogenic properties, VLPs have been explored as an alternative to inactivated vaccines (129). Most VLPs do not contain genetic material but instead are composed of immunogenic proteins such as the viral capsid. The advantage of this platform over subunit vaccines is the improved immunogenicity from using the native confirmation of the virus particles (129). VPL strategies that have been tried for RVFV include its glycoproteins and/or nucleocapsid protein (130-132). Applications of VLP vaccines are currently under evaluation for veterinary use.

The need for DIVA-compatible vaccines

For transboundary animal pathogens, DIVA-compatible vaccines are critical for disease control while allowing areas to maintain "disease-free" status. FMD, avian influenza (AI), and classical swine fever (CSF) are examples of transboundary animal diseases that demonstrate the need for DIVA vaccines with companion diagnostic tests for their eradication (133). The ability to differentiate infected from vaccinated animals has a strong economic incentive. For example, DIVA-compatible vaccines for FMD offer an alternative to the "stamping-out" method of depopulating all clinical animals and potentially exposed animals despite vaccination status. Implementing a DIVA-compatible vaccination protocol can minimize agricultural losses. However, DIVA-compatible vaccines and their companion diagnostic tests will need to be accepted by both livestock producers and agricultural trade partners globally to be successful in disease control (134). RVFV vaccine platforms such as the NSs/NSm deletion mutant vaccines and glycoprotein subunit vaccines are currently under evaluation to determine their efficacy as DIVA vaccines in livestock. Nonetheless, there is a lack of available DIVA-compatible companion diagnostic tools to accompany these candidate vaccines. The continuing development and evaluation of DIVA-compatible vaccines in conjunction with companion diagnostic tests should be emphasized to control the spread of RVFV.

Diagnostic techniques

Because RVFV shares similar clinical signs to many other pathogens, accurate diagnosis is critical during an outbreak. In the U.S. and Europe, there is a need to equip national and public health labs with diagnostic tests to rapidly identify viral hemorrhagic pathogens such as RVFV, Ebola, and anthrax. In Africa, cost-effective assays to identify pathogens and minimize their

agricultural impacts are needed when sudden death of neonates or abortion storms in livestock are observed. In these situations, multiplexing platforms are ideal to quickly rule out abortive diseases including Nairobi sheep disease, bluetongue, brucellosis, infectious bovine rhinotracheitits, and chlamydiosis. Additionally, RVF infections can be subclinical in animals and can easily be missed. It is important to have a versatile and specific assay to identify these cases so surveillance programs can monitor the status of disease in endemic areas. Several diagnostic assays are available for RVFV detection and can be categorized into virus isolation, antigen detection, nucleic acid detection, and antibody detection post-exposure. Early detection, versatility, and DIVA compatibility are important factors that currently drive the development of RVF diagnostic tests.

Virus isolation

RVFV can easily be isolated from whole blood and serum during its viremic phase (135). Additionally, infected liver, brain, spleen, and aborted fetuses are commonly used for RVFV isolation. Historically, *in vivo* virus isolation was performed by intracerebral injection of infected tissue homogenate into suckling mice. *In vitro* options have replaced this method due to animal welfare issues and biocontainment requirements. Several cell lines can be used for *in vitro* isolation including *Drosophila* (136), African green monkey kidney cells, baby hamster kidney (BHK), human fetal lung fibroblast cells (MRC-5), and various mosquito cells (137). RVFV is known for its consistent cytopathic effect (CPE) (138). CPE can be seen as early as 12-24 hr post infection (hpi) (2). As a routine diagnostic test, virus isolation is used less due to the biocontainment requirements. Therefore, recent diagnostic development has focused on safer alternative methods such as antigen detection.

Antigen Detection

An important technique for detecting RVFV antigens within tissue samples is immunohistochemistry (IHC). The technique enables visualization of antigens within the context of the wider tissue architecture including its histopathology and is valuable in both diagnostic testing and pathogenesis studies, including retrospective ones. The major organs in which RVFV antigens are typically found include liver and spleen. Viral antigens can also be found in brain, kidneys, lymph nodes, lungs, heart, adrenal glands, reproductive tract, skin, gall bladder, tongue, and the gastro-intestinal tract (33, 64, 139, 140). Additionally, the placenta from infected animals is usually positive by IHC although no lesions are seen (34). Finally, aborted fetuses contain detectable virus in visceral organs and brain (34, 141). It is important to note that detection of RVFV induced necrosis by histology does not guarantee detection of antigen by IHC in infected animals. This can be due to heterogeneous distribution of virus in infected tissues, timing of tissue collection in relation to disease progression, or the tissue quality at time of testing.

Some of the first RVFV studies with IHC used hyperimmune ascites fluid to detect RVFV antigen in tissues. Van der Lugt, et al. showed extensive hepatic necrosis in lambs as early as 18 hpi (64). Their IHC had no cross-reactivity with Wesselsbron virus in newborn lambs with severe hepatic disease. Shieh, et al. used monoclonal antibodies (mAbs) and polyclonal antibodies (pAbs) to determine the distribution of antigen in RVFV infected tissues from animals and humans (142). Their study concurred with previous findings that show RVFV replication occurring primarily in hepatocytes. Recently, IHC was developed using a polyclonal rabbit anti-RVFV N antibody. The assay correlates strongly with reverse transcription polymerase chain reaction (RT-PCR) and is more sensitive than virus isolation with experimental samples (143). In testing RVFV naturally infected tissues from South Africa, polyclonal hyperimmune mouse

ascites fluid was used as the primary antibody for IHC. This IHC had high specificity when compared to histological results and quantitative reverse-transcription PCR (RT-qPCR). A few false positive results were reported due to severe tissue autolysis potentially causing a loss of epitopes for detection (68). Therefore, care must be taken in the collection and treatment of tissues for accurate detection by IHC. A standardized RVFV antigen-detection protocol is needed to provide consistent and accurate results.

Nucleic acid detection

Nucleic acid detection offers a rapid testing method for RVF viral RNA. This is important for quickly identifying the virus and providing appropriate treatment and control measures. PCR and *in situ* hybridization (ISH) are popular nucleic acid detection techniques for viral pathogens. Several PCR tests have been developed to detect RVFV RNA including RT-PCR, macroassay RT-PCR, RT loop-mediated isothermal amplification (RT-LAMP), and recombinase polymerase amplification (RPA). An agarose gel-based RT-PCR and a real time RT-PCR for confirmation of samples infected with RVFV are recommended by the OIE terrestrial manual (2). To date, ISH has not been developed as a diagnostic tool for RVFV but it would offer an alternative method for testing for RVFV in tissues.

Polymerase chain reaction

Molecular detection by PCR has proven to be extremely valuable early in outbreaks for rapid diagnosis. It enables the identification of RVFV during an outbreak. Additionally, viral load can be determined, which aids in determining proper treatment courses for severe cases (144). PCR can be used for testing mosquito pools to better monitor disease spread and vector distribution (145, 146) and can be used as a DIVA-compatible companion test (147).

Several RVFV quantitative RT-PCR assays have been developed to provide molecular-based testing (143, 148-150). To extend the testing capability, real time RT-PCR has been designed in multiplex format to detect high consequence pathogens including RVFV, filoviruses, flaviviruses, variola virus, and vaccinia virus (151, 152). Another multiplex assay was developed for the differential diagnosis of RVFV, peste des petits ruminants, bluetongue, and rinderpest in ruminants using dual-priming oligonucleotides (153). Multiplexing molecular assays enables rapid screening of multiple pathogens in parallel using one sample. For example, an adaptation of macroarray-based technology and multiplex PCR was recently established to detect 29 pathogens that cause fevers or nervous system damage including RVFV commonly found in Africa (154). Lastly, a real time RT-PCR has been applied in a multiplex format to test multiple RVFV genes simultaneously, which enables for internal confirmation of positive samples (147). Additionally, this assay has been demonstrated to be DIVA-compatible with the NSs deletion Clone 13 vaccine strain. Multiplexing RT-PCR assays are valuable tools for both endemic and non-endemic areas.

There is a demand for molecular tests that can rapidly detect RVFV in the field during an outbreak. Techniques like isothermal amplification RT-LAMP and recombinase polymerase amplification (RPA) methods have been used for RVFV detection in the field. RT-LAMP has been used to amplify the RVFV L segment for blood and tissue and shows sensitivity comparable to real-time RT-PCR (155). Additionally, no cross-reactivity with other arboviruses was observed and it is in 100% agreement with virus isolation (156). Interpretation of results is simplified to visualizing a color change. A disadvantage to using RT-LAMP is the challenge of designing multiple primers including two inner, two outer, and two loop primers. An alternative isothermal amplification system is RPA. This technique uses dried and pelleted reagents making

it advantageous for field use. An RPA test designed to amplify RVFV S segment showed a high sensitivity of 10 molecules per reaction and a rapid assay time of 8 minutes (157). The use of isothermal amplification is ideal for field testing and rapid screening in outbreak situations.

In situ hybridization

In situ hybridization is a valuable tool for identifying nucleic acids of infectious pathogens accurately within tissues. The technique can be applied in both surveillance programs when livestock abortions are being reported and for research purposes. As a research tool, ISH can help improve understanding of viral pathogenesis in specific host tissues, which in turn can assist in vaccine and therapeutic development. For example, fluorescence ISH has been used to investigate the role of the NSs protein and its interactions with the host on a cellular level (158, 159). Additionally, fluorescence ISH using RNA probes has been used to visualize the packaging of RVFV genome segments (160). Conversely, ISH has not been used to detect RVFV RNA in tissues, specifically formalin-fixed, paraffin-embedded (FFPE) tissues. Testing of FFPE tissues offers a safe means of handling Select Agents and enables analysis of archived samples. Currently, RT-PCR (Upreti, et al., unpublished) and IHC methods are used to detect RVFV in FFPE tissues at Kansas State University but not ISH. RVFV RNA detection using ISH would be a valuable tool for virus confirmation in tissues outside biocontainment and offer an accurate tool in pathogenesis studies.

Antibody Detection

Detection of antibodies post-exposure enables the detection of the host immune response to disease. For RVFV, serological assays that detect ongoing or previous infections are important in surveillance programs and therapeutics development. Several assays have been developed for the detection of IgM and IgG antibodies against RVFV in animals and humans. The first RVFV

serological tests include agar gel immunodiffusion, hemagglutination inhibition, radioimmunoassay, immunofluorescence, and complement fixation (161). These assays are not commonly performed now due to technology advancements that provide for more rapid, safe, and accurate results. The virus neutralization test and enzyme-linked immunosorbent assay (ELISA) have become the primary serological assays for RVFV detection.

Plaque Reduction Neutralization Test

The plaque reduction neutralization test (PRNT) is a virus neutralization test that is extensively used for RVFV antibody detection. PRNT is considered to be the gold standard for RVFV diagnosis because of its high specificity in detecting RVFV neutralizing antibodies (2). Therefore, PRNT has become an important assay for testing serum from any species exported internationally. The standard PRNT for RVFV uses an 80% neutralization cutoff (162). A study reports the detection of neutralizing antibodies as early as 3 dpi in sheep (161). Yet, as seen in several challenge studies with sheep and cattle, a strong rise in titer is not detected until 6-7 dpi (66, 140). Unfortunately, PRNTs are laborious and time consuming thus non-ideal in outbreak situations beyond acting as a confirmatory test. Furthermore, because of the use of live virus the assay can only be performed in endemic countries or in biocontainment laboratories. This has led to a need for alternative serological tests that can rapidly screen samples and does not require handling of live virus.

Enzyme-linked immunosorbent assay

As an alternative to PRNTs, ELISAs offer a rapid, sensitive, and safe platform for the detection of RVFV. Both IgM and IgG ELISAs have been developed for human and animal testing. Initial ELISA development used whole virus and could detect antibodies earlier than other traditional serological methods. Nonetheless, high background was often observed despite

efforts to optimize reagents (161). ELISA platforms have since improved and diversified. IgM ELISAs can be used to detect antibodies as early as 4 dpi (9, 163-165). Therefore, these ELISAs are ideal for detecting RVFV early in the time-course of infection. IgG ELISAs detected a rise in antibodies as early as 5 dpi (9, 165). IgG ELISAs require testing of paired serum samples to distinguish active from past RVFV exposures. Current testing standards require a four-fold rise in antibody titer to confirm positive samples. The OIE Reference Laboratory in South Africa uses two ELISA tests: an IgM capture ELISA and an indirect IgG ELISA using the recombinant RVFV N protein (2). Detection of both IgM and IgG antibodies against RVFV by ELISAs is valuable for surveillance and demonstrating freedom from disease (166).

Several RVFV ELISA formats have been developed and validated. The first ELISA tests used whole cell lysate or purified protein as the target antigen (163, 167-169). Due to biosafety risks and the high production costs of these formats, there was a shift to developing ELISAs using recombinant proteins. The RVFV N is a strongly immunogenic and relatively easy to produce (165), and therefore, used frequently in indirect ELISAs for IgG antibody detection (170-172). Other RVFV recombinant antigens have also been developed including the glycoproteins Gn and Gc (173, 174) and the nonstructural protein NSs (175). The Gn and NSs targets have been shown to be immunogenic and offer a means for multiplex detection of RVFV antibodies. Currently, recombinant-based antigen ELISAs offer sensitive testing in non-endemic countries but most assays still require further validation and quality control for routine testing in national reference labs.

An important consideration influencing current serological assay development for RVFV is DIVA compatibility. To date, only a few DIVA-compatible RVFV ELISAs have been developed. McElroy et al. developed an indirect ELISA using recombinant N and NSs to be

compatible with NSs-deleted vaccine candidates (175). However, using a serological assay to detect the lack of antibody detection to NSs may lead to false negative results due to the variable antibody response to NSs between individuals (176). Additionally, anti-NSs antibodies are detected later during infection compared to anti-N antibodies (176). More recently, RVFV glycoproteins were shown to be DIVA compatible by ELISA when testing a candidate RVFV Gn/Gc subunit vaccine (177). DIVA detection by serological methods look promising; yet individual ELISAs are needed to test each RVFV antigen making the process laborious for routine testing. There is a need for a multiplexing serological assay that can simultaneously detect antibodies to several RVFV targets and allow high-throughput analysis.

Gaps in Knowledge

In 2017 the World Health Organization listed RVFV as one of the top ten emerging diseases that lack sufficient preventative and control measures (178). The advancements in vaccine and diagnostic technology are growing rapidly, yet there are a few validated diagnostic assays for rapid detection and control of RVFV. With recent changes in the regulations for the movement of infectious agents and samples, it is now more difficult to ship samples to laboratories equipped to test infectious samples (179). Therefore, there is a need to safely screen and confirm samples on the local level. The aim of this dissertation is to provide new diagnostic tools for testing various RVFV samples in both endemic and non-endemic countries.

DIVA-compatible vaccine strategies are critical in controlling transboundary animal diseases (134). Yet, there is a lack of DIVA-compatible companion diagnostic tools to accompany candidate RVFV vaccines. Serological testing using ELISAs can detect differences in antibody responses to a DIVA vaccine and a wild-type strain. However, ELISAs can only

detect antibodies against one antigen at a time, which can make testing cumbersome when testing for antibodies against multiple antigens. In contrast, a bead-based multiplex assay enabled the simultaneous detection of antibodies against the Gn and N protein, demonstrating the DIVA compatibility of the assay with subunit-based vaccines (180). Such assays still need validation with experimental and field samples for routine use.

Tissue samples are important in RVFV detection for they are typically the first thing acquired and submitted to a laboratory during an outbreak. Nonetheless, infected tissues must be handled with care due to their zoonotic risk. FFPE tissues offer a safe and versatile method for the diagnosis of Select Agents such as RVFV. PCR, IHC, and ISH are common detection methods for testing FFPE tissues. ISH is a rapidly growing technique and a powerful tool that provides nucleic acid detection in a histological context; yet, there is no ISH for the detection of RVFV RNA in FFPE tissues. Furthermore, to our knowledge, ISH has not been used as a DIVA-compatible assay to detect RVFV vaccine strains in tissues.

To develop effective vaccines and therapeutics against RVFV, there needs to be a better understanding of the virus and its interactions within the host. RVFV tissue tropism and pathogenesis are still poorly understood (52, 181). This is in part due to a lack of diagnostic tools needed to perform these studies. MAbs and pAbs have been developed against several RVF antigens including N, Gn, Gc, and NSs (9, 143, 182-184). Several mAbs against N, Gn, and Gc are readily available. However, these antibodies have not been evaluated for routine use in methods like IHC, western blot (WB), and immunofluorescence (IF). Access to robust working protocols and commercial antibodies would provide continuity in RVFV research.

Dissertation Objectives

This dissertation addresses the gaps in RVFV diagnostics. We describe the development of both a multiplexing serological and a molecular detection method for RVFV. We also provide protocols for RVFV antigen detection using readily-available antibodies. In Chapter 2, the objective is to evaluate a bead-based multiplexing immunoassay for the detection of antibodies against multiple strains of RVFV and its application as a DIVA compatible assay. We demonstrate that RVFV can be safely tested outside of biocontainment using recombinant protein targets to detect IgG and IgM antibodies against RVFV. Furthermore, the multiplexing capability of the assay enables the detection of DIVA compatibility with appropriate candidate RVFV subunit vaccines. In Chapter 3, we develop an ISH assay using RNAscope technology to detect RVFV RNA. We demonstrate the ability to detect RVF viral RNA in FFPE tissues, which is of great value in pathogenesis studies and as a confirmatory diagnostic test. Moreover, we develop a multiplexing RVFV ISH assay that is DIVA compatible with multiple RVFV gene deletion vaccines. Finally, in Chapter 4, we evaluate the suitability of several mAbs and pAbs against various RVFV antigens for standard immunodetection procedures. We expect these new diagnostic tools to be comparable in accuracy to current methods for RVFV detection. All the diagnostic tools developed and evaluated are critical tools for the control of RVFV and support vaccination strategies in both endemic and non-endemic areas. More importantly such work can be applied to the development of countermeasures for other high-consequence animal diseases.

Chapter 2 - Evaluation of Fluorescence Microsphere Immunoassay for the Detection of Antibodies to Rift Valley Fever Nucleocapsid Protein and Glycoprotein

Preface

The results of this chapter have been accepted for publication in the Journal of Clinical Microbiology under the title:

Evaluation of Fluorescence Microsphere Immunoassay for Detection of Antibodies to Rift Valley Fever Virus Nucleocapsid Protein and Glycoproteins

I.K. Ragan[1], A.S. Davis[1], D.S. McVey[2], J. A. Richt[1], R.R. Rowland[1], and W.C. Wilson[2]

[1]Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS, USA; [2] Arthropod-Borne Animal Diseases Research Unit, USDA, ARS, Manhattan, KS, USA

Introduction

Serological testing is a useful tool for the global surveillance of infectious diseases. It offers an economical and accurate means to detect disease status in animals. Advancements in diagnostics are replacing single-pathogen point-of-care tests with multiplexing platforms that can simultaneous and rapidly screen for several biomarkers (185). Until recently, applications of multiplexing immunoassays have lagged due to the complexity in coupling chemistry, poor antigen stability, susceptibility to cross-reactivity, and the difficulty in validating them for diagnostic use compared to singleplex assays like enzyme-linked immunosorbent assays (ELISAs) (186). Yet, the demand for low cost, high-throughput data collection and a more holistic approach to disease management will push the advancement of multiplexing technology.

Multiplexing Immunoassays

Multiplexing immunoassays are divided into two categories: planar and suspension assays. In general, they use antibodies or proteins as capture ligands immobilized on a solid surface to detect targets. Microarrays are an example of a planar multiplexing immunoassay offering high-throughput analysis of biomarkers. They can use proteins anchored in high-density microspots to capture target molecules, enabling a large number of targets to be screened with high sensitivity using a small sample volume (187, 188). Current technology contains up to 21,120 capture proteins, which is used for antibody validation (189). Yet, identifying multiple highly-specific capture proteins and the coupling of the proteins in correct orientation on slides can be challenging (190, 191). In contrast to planar assays, suspension assays use beads coupled to capture ligands to detect targets in liquid suspension. A population of beads containing the same capture ligand enables multiple independent measurements, which improves assay

precision compared to planar immunoassays (188). Moreover, new pathogen targets can be added to existing platforms without needing to revalidate the entire assay. Because of the dynamics of emerging infectious pathogens, bead-based suspension assays are advantageous over planar assays for their flexibility and their ease in incorporating new targets.

The concept of bead-based suspension assays came about in 1970's with the invention of flow cytometry to sort cells (192). By the 1990's the bead-based technology was used to detect nucleic acids, and proteins (193). Each bead can be bound to a different capture ligand that detects targets, also known as analytes, in samples. By being in suspension, analytes have more surface area to interact with the ligand compared to planar assays. Unfortunately, suspension-based multiplexing assays are prone to cross-reactivity when proteins cross link as the beads remain in suspension (188). Therefore, assay optimization must address cross-reactivity of (1) analytes to non-target analytes, (2) detection antibodies to non-target analytes, and (3) analytes to detection antibodies when multiplexing (194). Once validated procedures and controls are established, the bead-based suspension assay is a powerful tool for long-term routine testing in diagnostic laboratories.

Luminex® Technology

The most well-known and widely used bead-based multiplexing technology is Luminex® using xMAP technology®. The technology has the ability to multiplex up to 500 analytes (195) but latest validated assays only evaluate up to 30 analytes (196, 197) . Two instrument systems are available; a flow cytometry-based system and a magnetic bead-based system using charge-coupled device (CCD) cameras. The latter system, MAGPIX®, offers a more compact and robust multiplexing platform compared to the flow cytometry systems. The system uses colored

magnetic beads that are covalently coupled to capture antigens that bind to analytes in liquid suspension. A CCD camera identifies the color-coded beads and detects a secondary fluorescent conjugate that is attached to the target antibody in an indirect format (Fig 2.1). The technology is advantageous for foreign animal disease surveillance where routine testing of multiple pathogens simultaneously will decrease the cost, time, and sample volume needed (194). The MAGPIX® system has been proven for the detection of high-consequence viral pathogens like RVFV (180, 198), influenza (199), Ebola, and Lassa virus (200). The RVFV assay has yet to be validated for routine testing.

Serological testing of RVFV

Traditional serological detection for RVFV include complement fixation, hemagglutination inhibition, immunodiffusion, virus neutralization test (VNT), and ELISA (161). VNTs and ELISAs are the more commonly used tests for disease outbreaks and surveillance studies. VNTs are highly specific and are the current gold standard serological method for RVFV (2). Nonetheless, VNTs are labor intensive, time consuming, and require appropriate biocontainment unless using a non-virulent strain (201, 202). ELISAs offer a rapid and safe means to detect antibodies to RVFV in animals and humans. Although ELISAs are sensitive and reliable screening tests, they can only detect one analyte at a time.

Bead-based suspension assays allow for simultaneous detection of antibodies to several antigens of a single virus, consequently increasing the specificity of the assay. The assays can also be used as tests to differentiate infected from vaccinated animals (DIVA), which has been previously demonstrated with viral pathogens like avian influenza, foot-and-mouth, and West Nile viruses (203-205). A RVFV bead-based suspension assay has been previously developed to

simultaneously detect antibodies against RVFV N and Gn but the diagnostic accuracy of the assay was not established (180). Currently, there are no validated tests for the detection of RVFV antibodies that differentiate infected from vaccinated animals. The versatility and DIVA compatibility, with minimal impact on diagnostic accuracy when multiplexing, makes the bead-based suspension assay for RVFV testing a promising alternative to traditional serological methods.

We evaluate a bead-based suspension assay, alternatively called fluorescence microsphere immunoassay (FMIA), for the detection of RVFV antibodies against several antigen targets in sheep and cattle. The targets included the N protein, a truncated glycoprotein Gn (minus transmembrane domain), NSs, and NSm. The assay was used to detect several RVFV strains and was compared side-by-side to the VNT. We also demonstrated the use of FMIA as a DIVA-compatible platform for a candidate RVFV Gn/Gc subunit vaccine.

Materials and Methods

Sera Samples

A total of 154 cattle and 272 sheep sera from prior experimental RVFV animal studies were used for the evaluation. These included sheep and cattle inoculated with vaccine strain MP12 (97), wild-type strains SA01-1322 (SA01) and Kenya-128B-15 (Ken06) (206, 207), and in the presence or absence of a candidate Gn/Gc subunit vaccine (106, 177). These sample sources are summarized in Table 2.1. All studies were conducted under biosafety level 3-enhanced conditions at the Biosecurity Research Institute (Manhattan, KS). Sera were heat inactivated and safety tested for further diagnostic evaluation at biosafety level 2 using a modified method that successfully inactivated high titer samples. Briefly, all sera were inactivated by adding 0.25%

Tween-20 at a dilution of 1:10 to the serum and heating samples to 60˚C in a water bath for 2 hours (hr) as previously described (207).

Additional sera from an experimental study of a RVFV MP12-NSm deletion vaccine candidate in sheep were tested. Briefly, a reverse genetics system using the MP12 strain produced a vaccine virus in which a portion of the NSm gene has been removed (arMP12ΔNSm21/384) (102). A total of 119 sheep samples were tested for the evaluation.

RVFV challenge sera was obtained from an experimental challenge with wild-type Egyptian isolate ZH501 (66). Sheep and cattle were inoculated and kept up to 35 dpi to assess antibody production. A total of 4 sheep and 7 cattle samples were tested. These samples served as positive-control sera for the FMIA.

Sera from animals never exposed to RVFV were used as the negative sera set, which included 165 sheep and 325 cattle samples. All samples were obtained from animals born and raised in the United States. The sera were heat inactivated following the protocol described above.

Production of Recombinant Proteins

RVFV Gn was produced from the coding sequence taken from the RVFV MP12 strain (GenBank DQ380208) and modified to remove the transmembrane domain. The sequence was synthesized (IDT, Coralville, IA) and cloned into a pHUE expression vector (208), then transformed into BL-21 *Escherichia coli* (*E. coli*) cells (New England Labs; Coralville, IA). Gene expression was induced with 1 mM isopropyl-beta-D-thiogalactoside in LB culture medium with constant shaking for 4 hr at 37˚C. Bacterial cells were harvested by centrifugation then purified using the PrepEase Histidine-Tagged Protein Purification High Yield Midi Kit

(Affymetrix, USB Corp.; Cleveland, OH) under denaturing conditions. Purified protein was then precipitated to remove urea and exchanged with 1x phosphate buffered saline and 0.1% SDS, as previously described (209). The precipitated protein was concentrated using Amicon Ultra-2 ml Centrifugal Filters (EMD Millipore Corporation; Billerica, MD) with a molecular size cutoff at 30kDa. The protein concentration was measured by Bradford protein assay (Bio Rad, Hercules, CA). Purified protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 10% and stained with Coomassie Brilliant Blue (Bio-Rad; Hercules, CA).

Purified recombinant RVFV N, NSm, and NSs proteins were produced in an *E. coli* expression system as previously described (198). Before use, proteins were analyzed by SDS-PAGE 10% and stained with Coomassie Brilliant Blue (Bio-Rad; Hercules, CA) to visualize purity and integrity.

Non-RVFV recombinant proteins were used as negative-control coupled beads to account for nonspecific binding of antibodies. Porcine circovirus type 2 nucleocapsid (PCV2) was selected to represent a non-RVFV viral nucleocapsid protein that was expressed in an E. coli expression system using the pHUE vector and purified using the PrepEase Histidine-Tagged Protein Purification High Yield Midi Kit as described above under native conditions. Purified protein was then analyzed by SDS-PAGE.

All proteins were analyzed by western blot (WB) to assess recognition of antigens by mAbs and pAbs. Briefly, 10 ng of each recombinant protein was separated by SDS gel electrophoresis then transferred onto polyvinylidene difluoride (PVDF) membrane by electroblotting using the TransBlot Turbo Transfer Pack on the TransBlot Turbo Transfer System (Bio Rad, Hercules, CA). The membranes were blocked with a protein-free blocking buffer (G-

Biosciences; St. Louis, MO) at 4˚C overnight with rocking. The membranes were washed three times using PBS with 0.1% Tween-20. To visualize reactivity of proteins against ruminant sera, membranes were incubated with a strong positive sheep sera experimentally infected with RVFV ZH501 strain (66) diluted 1:100 for 1 hr at room temperature (RT). The cross-reactivity of proteins was also tested using negative lamb sera diluted 1:100. After washing, rabbit anti-sheep IgG (H+L)-HRP conjugate (Bio-Rad; Hercules, CA) diluted 1:20,000 was applied for 1 hr at RT. To visualize the reactivity of RVFV recombinant proteins against mAbs, primary antibodies against N and Gn were acquired from Maine Biotechnology Services (MAB240P) (clone 09F04) (Portland, ME) and BEI Resources (NR43190) (clone 4D4) (Manassas, VA), respectively. All mAbs were diluted 1:1,000 and incubated for 1 hr at RT. After washing, a peroxidase AffiniPure donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories; West Grove, PA) secondary antibody was added at a 1:100,000 dilution for 1 hr at RT. After a final wash, Clarity ECL Blotting substrate (Bio-Rad; Hercules, CA) was added for 5 minutes (min) for the detection of proteins. The membrane was visualized using the ChemiDoc Mp Imaging System (Bio-Rad; Hercules, CA).

Conjugation of antigens to Luminex carboxylated beads

Recombinant proteins were covalently coupled to MagPlex Microspheres® (Luminex Corp.; Austin, TX) by using a two-step carbodiimide reaction as previously described (198). Briefly, 6.25 x 10^6 microsphere beads per target were activated and then incubated with their respective recombinant protein for 3 hr at RT with rotation. Coupled bead sets were stored in blocking buffer (1% fish gelatin in PBS with 0.05% Tween20 and 0.05% sodium azide, pH 7.4) overnight to reduce non-specific binding of antibodies to the beads. The efficiency of the protein

coupling reaction was confirmed using a mouse Penta-His antibody, BSA-free (Qiagen; Germantown, MD) according to the bead manufacturer's instructions. Bead sets were stored at 4°C and used within 4 weeks to minimize signal loss due to protein degradation. To minimize photo-bleaching effects on the beads, all coupling reactions, storage, and use of beads were done in reduced lighting. The final concentration of each coupled bead set was manually counted using a hemacytometer.

Optimization

The amount of RVFV protein coupled to the beads was optimized to identify the strongest signal against positive sera for each coupled bead set. The beads were coated and tested with various protein concentrations, including 12.5, 25, 50, and 100 µg/mL per antigen. The antigen concentration that provided the strongest signal-to-noise ratio for all targets was used for testing samples.

The FMIA platform was optimized to determine the ideal concentrations of sera and reagents. A well-characterized strong positive-control serum and a negative-control serum were serially diluted in a checkerboard fashion to optimize the signal-to-noise ratios. Serum, antibody, and fluorescent reporter dilutions were selected based on ideal signal-to-noise ratio for both the N and Gn bead targets.

A set of beads coupled to a non-RVFV viral recombinant antigen, PCV2, was included to account for non-specific binding of antibodies to the recombinant antigens coupled to the beads. A bead set with no coupled antigen was included to account for background due to antibodies non-specifically binding to the beads. Each bead set was tested in singleplex format then in

multiplex format to identify any cross-reactivity between multiplexing bead sets and detection antibodies.

Fluorescence microsphere immunoassay

All FMIA tests used the Luminex MAGPIX ® System (Luminex Corporation; Austin, TX). All sheep sera samples were diluted 1:400 in assay buffer (1% fish gelatin in PBS with 0.05% Tween-20 and 0.05% sodium azide, pH 7.4). All cattle sera samples were diluted 1:200 in assay buffer. Samples were transferred to a 96-well round-bottom polystyrene plate (Corning, Inc; Corning, NY) and plated in triplicates at 50 µL per well. Control sera, which included a strong positive, a medium positive, a low positive and a negative serum sample, were added to each plate to serve as an internal quality control. An additional well with only the bead sets and assay buffer was added to each plate to calculate background signal. Each well received 50 µl assay buffer containing each bead set at a concentration of 2500 total beads per antigen. Plates were covered with foil to minimize light exposure then incubated 30 min at RT on a plate shaker set at 800 rpm. Using a magnetic bead separator, plates were washed three times using 190 µL of assay buffer per well. The anti-species secondary antibody was diluted in assay buffer and added at a volume of 50 µL per well and incubated for 30 min at RT. Ovine antibodies were detected with rabbit anti-sheep biotinylated IgG (Jackson ImmunoResearch Laboratories; West Grove, PA) diluted to 1 µg/mL and rabbit anti-sheep biotinylated IgM (MyBioSource; San Diego, CA) diluted to 1µg/mL. Bovine antibodies were detected with goat anti-bovine biotinylated IgG (Jackson ImmunoResearch Laboratories; West Grove, PA) diluted to 1µg/mL. The plates were washed as described previously then 50 µL of the fluorescent conjugate, streptavidin-phycoerythrin (SAPE) (Moss, Inc; Pasadena, MD) was diluted to 1 µg/mL and added to each

well. Plates were incubated for another 30 min at RT then washed. Beads in each well were resuspended in 100 µL assay buffer and the plate was analyzed on the MAPGIX using xPONENT version 4.2 software (Luminex Corporation; Austin, TX). Analysis of fluorescence was set to measure 100 beads per bead set per well. Results were recorded as median fluorescence intensity (MFI). Background signal detected from the blank well containing only beads and assay buffer was subtracted from each sample well. The average MFI was calculated for each triplicate set and then converted to sample/positive control (S/P) ratio to normalize results across plates. The S/P ratio was calculated by using the formula: S/P = (Mean MFI of test sample - Mean MFI of negative control) / (Mean MFI of high positive control – Mean MFI of negative control).

Virus Neutralization

For the validation of the FMIA, a plaque reduction neutralization test for RVFV was performed as described previously (106). Briefly, RVFV MP12 virus was diluted to approximately 50 plaque forming units (pfu) per 250 µL in Minimum Essential Medium (MEM) (Thermo Fisher Scientific, Grand Island, NY) containing 2% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO). Sera were serially diluted two-fold from 1:10 to 1:1280 in MEM with 2% FBS and 1% penicillin-streptomycin-Amphotericin B (Thermo Fisher Scientific; Grand Island, NY). The diluted sera were mixed at a 1:1 volume with diluted MP12 virus on a 96 well plate then incubated for 1 hr at 37°C. Virus-serum mixtures were then used to inoculate a confluent monolayer of Vero cells (ATCC® CCL-81TM) on 12-well plates. Plates were incubated for 1 hr at 37°C with gentle rocking every 15 min, then an overlay with 1% methylcellulose (Sigma-Aldrich; St. Louis, MO) and MEM was added to all wells and plates

incubated for 5 days at 37°C. The formation of plaques was visualized, and the plaques were counted after staining them with crystal violet fixative stain (0.5% crystal violet, 1% formaldehyde, ethanol, and glacial acetic acid in water) for 1 hr at RT. Neutralizing antibody titers were determined using an 80% neutralization cutoff, which corresponds to the reciprocal titer of the highest serum dilution at which the number of plaques is reduced by ≥80% compared to the MP12 strain virus control. A sample was considered positive if there was a detectable titer at or above the dilution of 1:10 to indicate exposure to the virus.

Statistics

Pearson's correlation coefficient was calculated to compare the singleplex versus multiplex FMIA platform and to compare the FMIA to VNT. Two-graph receiver operating characteristic (TG-ROC) was used for determining the FMIA cutoff values for each target. Youden's index was used to calculate diagnostic accuracy for each target using the formula (*J*)=[Sn+(Sp-1)]. The Np (1) and Gn (2) targets were tested in series and in parallel using the following formulas: Sn (series)= Sn1 x Sn2, Sn (parallel)= 1 – (1-Sn1) x (1-Sn2), Sp (series)= 1 – (1-Sp1) x (1-Sp2), Sp (parallel) = Sp1 x Sp2. A *t* test was used to analyze differences in antibody detection between three strains of RVFV used for experimental challenge. Analysis was done using Graph Pad Prism software (version 7.0) (Graphpad Software; Inc, La Jolla, CA).

Results

Expression and immunogenicity of protein targets

All FMIA panel proteins were produced, purified, and evaluated for antibody reactivity (Fig. 2.2 A). RVFV proteins were reactive against positive sheep serum at 28 dpi with the RVFV

ZH501 strain. The N showed the strongest reactivity followed by NSs, Gn, and finally NSm (Fig. 2.2 B). Negative control proteins were non-reactive. Negative lamb serum, tested against all proteins, was also non-reactive (data not shown). Further testing of N and Gn using anti-N and anti-Gn mAbs revealed that anti-N reacted strongly with the N protein but not with Gn (Fig. 2.2 C), while anti-Gn reacted with the Gn protein, and not with N (Fig. 2.2 D), albeit more weakly than anti-N and N. A similar examination of the NSs and NSm proteins was not possible due to lack of working antibodies. Overall, these results demonstrate the antibody reactivity of target proteins to be used for the FMIA.

FMIA optimization

Optimization of RVFV N and Gn protein concentrations revealed that 25µg/mL of each offered the strongest signal-to-noise ratio for both bead targets. When seeking the optimal serum dilution for detecting IgG and IgM antibodies, serial dilution of the sheep and cattle sera revealed a concentration-dependent MFI signal pattern (data not shown).

Initially, IgG antibody detection by FMIA in both sheep and cattle sera had high background. Therefore, subtraction of the MFI signals from PCV2 and the blank (negative control) beads from each sample accounted for the non-specific binding of primary antibodies to the bead-coupled recombinant antigen and to the beads, respectively. These two adjustments afforded a better interpretation of the RVFV antigen targets detecting antibodies in the sera.

Each bead set was tested in singleplex then in multiplex format against control sera to identify any cross-reactivity between bead sets (Fig. 2.3). The correlation coefficient for each bead set was high (R^2=0.99), demonstrating minimal cross-reactivity between bead sets. There was no statistical difference between the singleplex and multiplex format (*P* value >0.05).

The MFI signals for the NSs and NSm bead sets against ruminant sera were weaker when compared to the MFI signals from the N and Gn bead sets. Detection of antibodies against the NSs target was variable in known-positive samples making NSs a poor target for a screening assay. MFI signals against the NSm target were at or below assay cutoff in known-positive samples and by WB. Additionally, the NSm recombinant protein was only weakly reactive against positive sera (Fig. 2.2 B). Due to these issues that require additional optimization, the NSs and NSm bead sets were excluded from further analysis and evaluation of the FMIA focused on N and Gn targets.

Humoral immune response detection by FMIA

IgG antibody detection over time for the N target was demonstrated using samples from experimental challenge studies with three RVFV strains: SA01, Ken06, and MP12. There were no detectable IgG responses for any of the targets to mock-challenged animal sera (data not shown). Sheep IgG antibodies were detected as early as 4 dpi for SA01 and Ken06, and 7 dpi for MP12 (Fig. 2.4 A). Peak response occurred by 10 dpi for SA01 and Ken06, and 14 dpi for MP12. Cattle IgG antibodies were detected by 4 dpi for SA01, 5 dpi for Ken06, and 8 dpi for MP12 (Fig. 2.4 B). Peak response occurred at 21 dpi for SA01 and Ken06, and 14 dpi for MP12. Regardless of species, FMIA results for the N target were similar for SA01 and Ken06 samples. While the MP12 vaccine strain samples consistently produced a weaker MFI signal statistically, the finding was not significant.

As demonstrated with the N target, the Gn target was also detected by IgG subtype antibodies in the challenge studies. Compared to N, the Gn target had an overall lower MFI signal in both species (data not shown). In sheep samples, detection of IgG antibody production

occurred at 8 dpi in sheep samples for all strains. Ken06 had the strongest signal with a peak response at 14 dpi. For SA01, signal increased until 10 dpi then decreased throughout the rest of the study. As seen with the N target, MP12-induced antibodies overall showed the weakest signal of all the strains. In cattle samples, detection of IgG antibodies by the Gn target was variable and the background was higher. Overall, the N target was more sensitive than the Gn target in the detection of IgG antibodies earlier in an infection time course (P value < 0.05).

Detection of IgM antibodies against RVFV N and Gn targets was similarly tested in sheep sera. IgM antibodies were detected against the N target as early as 4 dpi for the SA01 and Ken06 RVFV strains (Fig. 2.5 A). Peak MFI signal occurred by 7 dpi then declined starting at 8 dpi for SA01 and 14 dpi for the Ken06 respectively. As with the IgG detection, Ken06 had a higher MFI signal than SA01on most time points, although the differences were not statistically significant. For the Gn target, a rise in IgM antibodies was detected at 6 dpi with a peak signal by 9 dpi and then a decrease in signal by 21 dpi (Fig. 2.5 B). Similar to IgG antibody detection, the detection of IgM by the Gn revealed an overall lower signal that of the N target. Cattle sera were not evaluated for IgM detection due to the high background noted in the IgG FMIA assay against the Gn target.

Comparison of FMIA vs. VNT

A total of 518 sheep and 447 cattle samples were tested by the gold standard assay, VNT. Statistical comparison of the FMIA and VNT showed the two assays to be highly correlated (R^2=0.89) when a comparison was made between the FMIA panel detecting IgG antibodies against the N target for sheep inoculated with wild-type strains (Ken06, SA01) (Fig. 2.6 A). Also, a good correlation (R^2=0.71) was seen when the VNT was compared to the FMIA for

detection of IgG antibodies against Gn (Fig. 2.6 B). There was a strong correlation (R^2=0.91) when a comparison was made against the N target with sheep inoculated with MP12, but the correlation was weaker against the Gn target (R^2=0.55).

Diagnostic accuracy

Cutoff MFI values for the Np and Gn bead targets were determined by TG-ROC analysis. All samples were classified positive or negative by VNT results. The Youden's Index (YI) was used to determine the "optimal" cutoff value for which Sensitivity (Sn) + [Specificity (Sp) – 1] is maximized (210). Table 2.2 summarizes the cutoffs, Sn, Sp, YI, and Area Under Curve (AUC) by each bead target in sheep and in cattle. Table 2.2 also contains the Sn and Sp for the two targets (N and Gn) when tested in series and in parallel using sheep samples.

DIVA compatibility with candidate RVFV vaccines

Sheep sera from a candidate Gn/Gc subunit vaccine study demonstrated the DIVA capability of the FMIA. The DIVA capability of the FMIA was not evaluated in cattle due to the lack of available Gn/Gc subunit vaccine cattle sera. In the sheep study, vaccinated and mock-vaccinated animals were readily separable (Fig. 2.7). The FMIA detected a rise in antibodies against Gn by 7 dpi in sera collected after the initial vaccination (Fig. 2.7 A). After a booster vaccination at 21 dpi, there was a stronger MFI signal against the Gn target. No antibodies against the N target were detected, as expected (Fig. 2.7 B). Mock-inoculated animals had no rise in IgG antibodies against either the Gn or N target. All animals were then challenged with a wildtype RVFV strain and monitored for 7 days. Sera from animals that were vaccinated had a higher MFI signal against the Gn target compared to the MFI detected in the mock-vaccinated

animals (Fig. 2.7 C) (*P* value<0.05). IgG antibodies were detected against the N target as early as 4 dpi in the mock-vaccinated animal sera and MFI values were higher compared to the vaccinated animal MFI signal levels, which remained unchanged after the challenge (Fig. 2.7 D) (*P* value<0.05). This difference in observed MFI demonstrated the FMIA's DIVA capability.

Discussion

FMIA is a growing technology that offers a versatile and rapid multiplexing platform for diagnostic testing and epidemiological studies. Yet there are limited reports about the use of FMIA in the detection of transboundary animal diseases (180, 204, 211-214). We demonstrate the ability of the FMIA assay to detect antibodies against RVFV recombinant antigens. We have previously developed the FMIA assay for RVFV N, Gn, NSs and NSm to detect antibodies in serum samples from experimental infection trials (198). However, during field trials, high background was noted in this assay. Subsequently, we modified and evaluated a new FMIA assay that focused on recombinant RVFV N and Gn proteins as antigenic targets for detection of IgG and IgM antibodies in experimentally infected ruminant sera.

Recombinant RVFV N is an ideal diagnostic target for serological tests since it is considered the immunogenic protein that induces early production of antibodies during infection (9, 215). Our study found that RVFV N to be highly immunogenic, with strong MFI signals for detecting both IgM and IgG antibodies. Diagnostic accuracy was high with sheep and even higher with cattle samples. There was a stronger correlation (R^2=0.89) between the FMIA N target and the gold standard VNT assay than between Np-based ELISAs and VNTs (171). Lastly, the timing at which antibodies against N were detected by FMIA supports what has been seen with recombinant Np-based ELISAs (9, 165, 171). Furthermore, the N target detected antibodies

earlier during infection compared to the secondary Gn target in the FMIA. Therefore, the N target is ideal for early disease screening of samples by FMIA.

RVFV glycoproteins are also important antigen targets, as they are the components of RVFV that are most exposed to the immune system during infection (166). The FMIA results for the Gn target against sheep sera were comparable to a Gn-based IgG ELISA (216). The FMIA Gn target correlation to VNT was weaker than that for N but still reasonable. This difference was due to the VNT detecting neutralizing antibodies earlier in the infection than were detected by the FMIA Gn target resulting in a higher false negative rate. It should be noted that the VNT titers for most of the Gn-FMIA negative samples were borderline positive titer (<1:40). The Gn bead set could not be fully evaluated for cattle samples due to high background, which has been previously noted in other serological assays when using cattle sera (D.S. McVey, personal communication). Future development and optimization of smaller Gn peptides with critical epitopes for antibody binding may reduce cross-reactivity of antibodies and offer a simpler way to produce antigen targets versus whole recombinant proteins (217, 218). Additionally, expression of Gn in a nonbacterial system may reduce cross-reactivity from bacterial contaminants as well as support posttranslational protein modification that may improve antigenicity as was seen in the report of van der Wal et al., in which the Gn was more antigenic than the N (174, 180).

When testing the N target and Gn target in singleplex, the Np target had high Sn and Sp, while the Gn target had high Sn but poor Sp. The YI and AUC values also demonstrates the higher accuracy of the N target compared to Gn. Most serum samples tested were collected at times of early antibody production, when antibodies against N are present but may be too early against Gn. Additional sera from later time points need to be tested to better evaluate the Gn

target. Overall, the cattle FMIA panel had higher MFI signals compared to the sheep FMIA panel, which resulted in higher assay cutoff value. Addressing the background issues with cattle sera may reduce the cutoff values to a comparable level with the sheep assay.

To evaluate the FMIA in multiplex, the N and Gn targets were tested in parallel and in series. The analysis was only done with sheep sera due to the high background observed with cattle sera. When testing in series the Sp improved to 98% compared to singleplex testing (92% for N, %67 for Gn). When testing in parallel the Sn improved to 99% compared to singleplex testing (94% for N, 89% for Gn). However, the Sp is greatly reduced in parallel testing. When testing the targets in multiplex, testing in series will offer a highly Sp assay ideal for confirmatory testing.

RVFV N and Gn are important as DIVA-compatible targets for a candidate RVFV glycoprotein-based vaccine. Recently, a candidate Newcastle disease virus vaccine expressing RVFV glycoproteins was used to evaluate a bead-based suspension array for differentiating vaccinated and non-vaccinated animals (180). Our study demonstrated that the FMIA is DIVA compatible with a candidate RVFV Gn/Gc subunit vaccine. Additionally, we were able to differentiate vaccinated and nonvaccinated animals prechallenge. DIVA vaccines and companion tests are important tools in control and eradication programs of high-consequence animal diseases. Further evaluation of our FMIA with other DIVA-compatible RVFV vaccines such as NSm deletion vaccine candidates is under way (102).

With several candidate nonstructural protein-deletion RVFV vaccines being evaluated, RVFV NSm and NSs are valuable diagnostic targets for companion DIVA assays. NSm serves as a DIVA marker in some recombinant RVFV vaccine candidates (16, 101, 102, 219). Nonetheless, NSm was a poor antigenic target in the FMIA due to a weak antibody response

when compared to N and Gn. Additional work may further optimize a recombinant NSm that could be used as an effective antigenic target on the FMIA panel, however, the host response to NSm may be too limited to be useful. Several NSs-deletion vaccines are being developed thus this protein could be used as a DIVA-compatible marker (16, 90, 103, 219). Recombinant NSs antigen was used as a target in the FMIA, and strong MFI signals were detected to antibodies against NSs. Yet, the antibody response was variable between infected animals similar to what has been reported with NSs-based ELISAs (176). Therefore, using NSs could be used as confirmatory RVFV target but not a reliable DIVA marker, except on the herd level. Further investigation is needed to understand the mechanism of the observed variable antibody responses of less immunogenic RVFV antigens to develop a diagnostic assay that can be applied for testing on the individual animal level.

The FMIA is expected to be more sensitive compared to traditional solid-phase assays like ELISAs because of the freedom for the antibodies to bind to epitopes in suspension (194). The current antigen targets demonstrated slightly reduced sensitivity. This may be due to the coupling of antigen to beads interfering with the antibodies accessing epitopes. Another factor may be the potential inactivation of epitopes during the carbodiimide coupling reaction (220). Introduction of spacers near epitopes of interest would enhance the ability of the protein targets to detect antibodies and improve antigen orientation. Evaluation of shorter peptides for RVFV should be done to identify important epitopes that can be used for the FMIA.

Future Directions

The challenge of validating biomarkers for multiplex testing is to characterize them for stability, sensitivity of detection, and the dynamic range of detection in multiplex format (190).

Ideally a larger sample set should be tested to validate the FMIA for RVFV detection. Due to increasing restrictions on Select Agent research, it may not be feasible to acquire additional experimental RVFV samples. The current focus for the assay is to validate with field samples from endemic countries. Recently, FMIA coupled beads were tested on naturally-infected ruminant sera from Kenya in collaboration with the International Livestock Research Institute. The results of the FMIA were compared to two commercial RVFV ELISAs (Lindahl and Ragan, et al., unpublished). Additional testing of field samples with VNT results will be needed to establish diagnostic accuracy of the FMIA when applied in endemic countries.

In addition to testing field samples, we are interested in evaluating the stability of conjugated-FMIA bead sets for use in endemic countries where optimal storage of bead sets may not be available. Currently we are evaluating the stability of conjugated beads for maximal storage time and optimal storage temperature (Ragan, et al., unpublished). Conjugated beads were regularly tested for one year to identify any changes in MFI signal. Beads were also stored at -20°C, 4°C and RT conditions to determine the optimal storage conditions. The results of the study will offer insight into the stability of conjugated beads and will optimize the detection of antibodies against RVFV.

Conclusion

We evaluated a multiplexing serological assay using recombinant RVFV N and Gn. The initial background seen during field trials has since been resolved and future evaluation of the assay will include additional field samples. The results demonstrate that the FMIA offers a high-throughput and versatile multiplexing tool for animal disease surveillance. This assay can be used for routine serological testing in endemic countries or can be incorporated into a foreign

animal disease early warning strategy in non-endemic countries. The RVFV N and Gn antigen targets could be combined into a larger multiplexing panel for screening several ruminant diseases such as bluetongue, Wesselsbron, peste des petits ruminants, and other abortive diseases. Lastly, the FMIA can be used as a DIVA-compatible assay for candidate RVFV subunit and recombinant vaccines.

Acknowledgements

RVFV MP-12-NSm deletion vaccine study sera were kindly provided by John Morrill, University of Texas Medical Branch through Doug Watts, University of Texas at El Paso. RVFV positive sera was provided by Hana Weingartl, National Centre for Foreign Animal Disease, Canadian Food Inspection Agency Winnipeg, Manitoba, Canada. The authors thank Maryka Smith (KSU) for technical support and Mal Rooks Hoover (KSU) for assistance in medical illustration. The views and conclusions contained in this document are those of the authors and should not be interpreted as necessarily representing the official policies, either expressed or implied, of the U.S. Department of Homeland Security or U.S. Department of Agriculture. This work was supported by the Kansas State NBAF Transition Funds, Kansas State University College of Veterinary Medicine, the USDA Agricultural Research project number 3020-32000-009-00D, and the U.S. Department of Homeland Security under Grant Award Number DHS-2010-ST-061-AG0001. No competing financial interests exist.

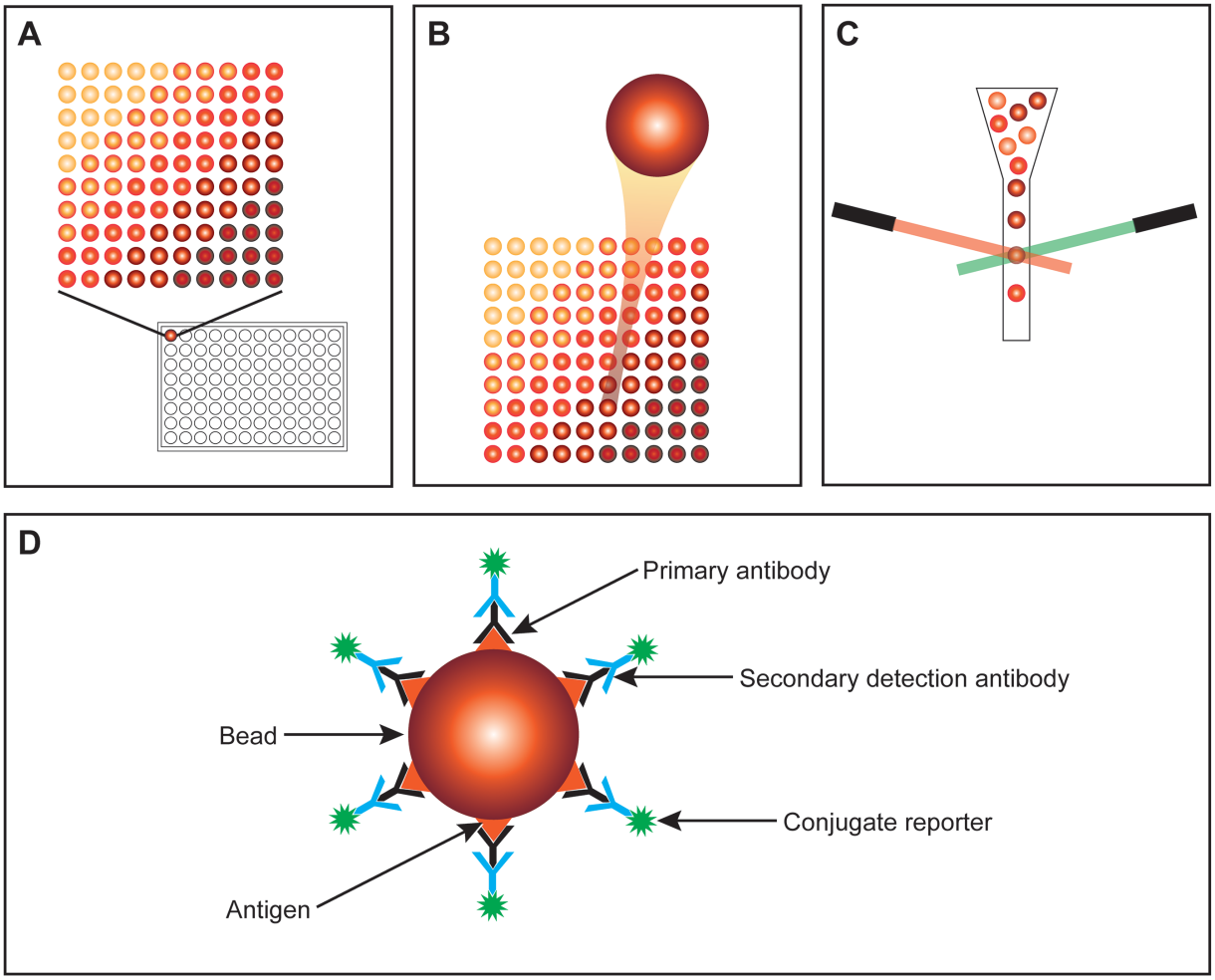

Figure 2.1 The FMIA utilizes color-coded polystyrene microspheres that can be coated with protein.

(A and B) The Luminex MAGPIX system has up to 50 spectrally distinct bead sets to allow for simultaneous detection of multiple biological targets in a single sample. (C) Once the target of interest is captured, each bead is individually read using a CCD camera. (D) RVFV recombinant proteins are covalently coupled to the beads and incubated with sera for the detection of primary antibodies. A biotin-labeled antispecies secondary antibody followed by a fluorescent conjugate (streptavidin-phycoerythrin) is added to detect the presence of primary antibody.

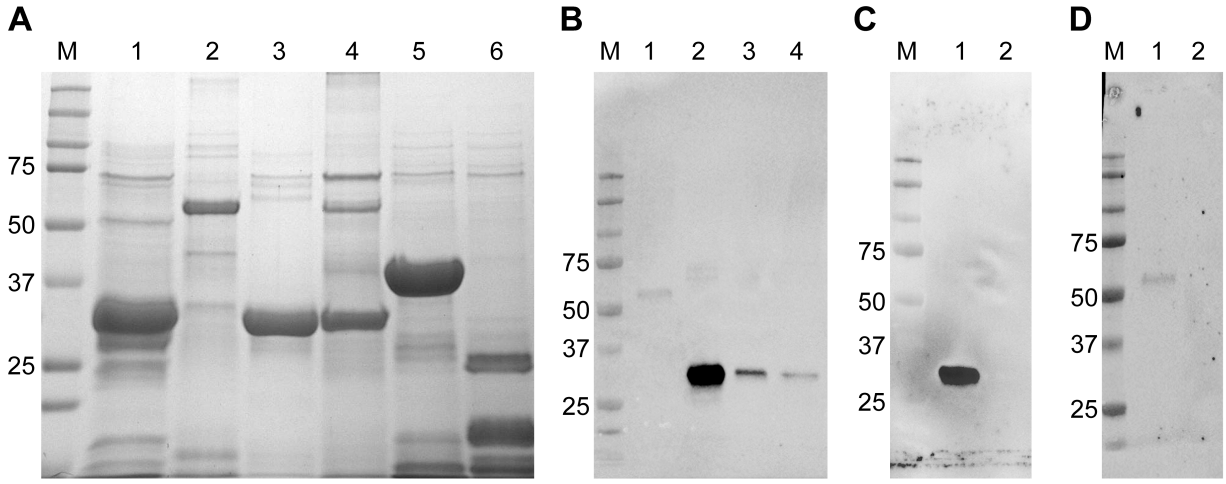

Figure 2.2 RVFV and negative control recombinant proteins used in FMIA.

(A) All proteins were analyzed by SDS-PAGE to visualize their integrity. Lane M, molecular size marker (in kilodaltons); lane 1, RVFV N (32kDa); lane 2, RVFV Gn (54kDa); lane 3, RVFV NSs (34kDa); lane 4, RVFV NSm (30kDa); lane 5, GFP (37kDa); and lane 6, PCV2 (22kDa). (B) WBs analyses were done to confirm the presence of the RVFV proteins. All four RVFV targets were probed with a positive sheep serum. Lane M is molecular size marker (in kilodaltons); lane 1, RVFV Gn; lane 2, RVFV Np; lane 3, RVFV NSs; lane 4, RVFV NSm. (C and D) N and Gn were further tested against mAbs. (C) Monoclonal MAB240P (clone Np09F04) was used against N. Lane 1, RVFV N; Lane 2, RVFV Gn. (D) Monoclonal NR43190 (clone 4D4) was used against Gn. Lane 1, RVFV Gn; lane 2, RVFV N.

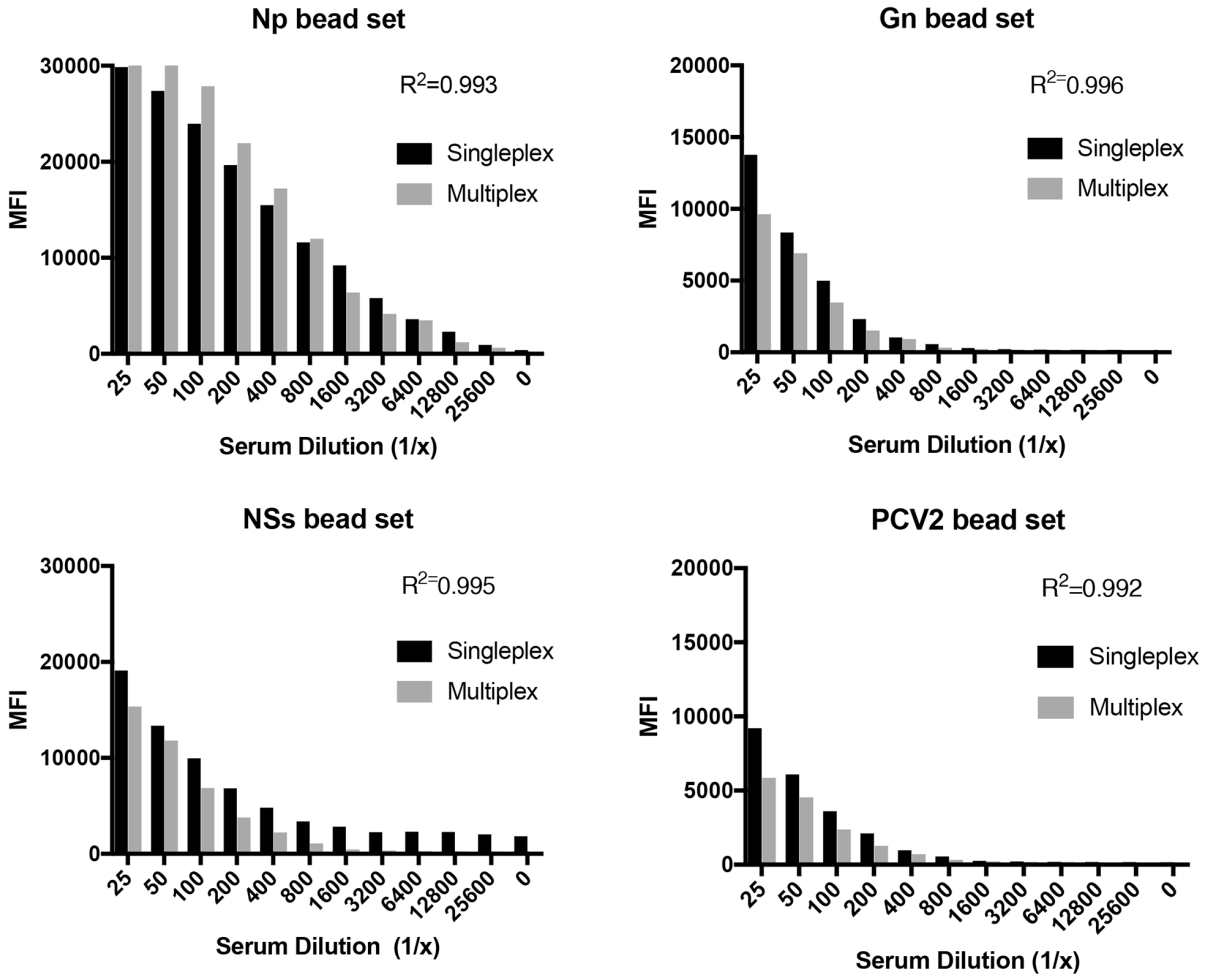

Figure 2.3 Comparison of singleplex and multiplex format of FMIA.

Each bead set was tested in singleplex then in multiplex format against positive sheep sera. The correlation coefficient (R^2) between the singleplex and multiplex format is provided for each bead set.

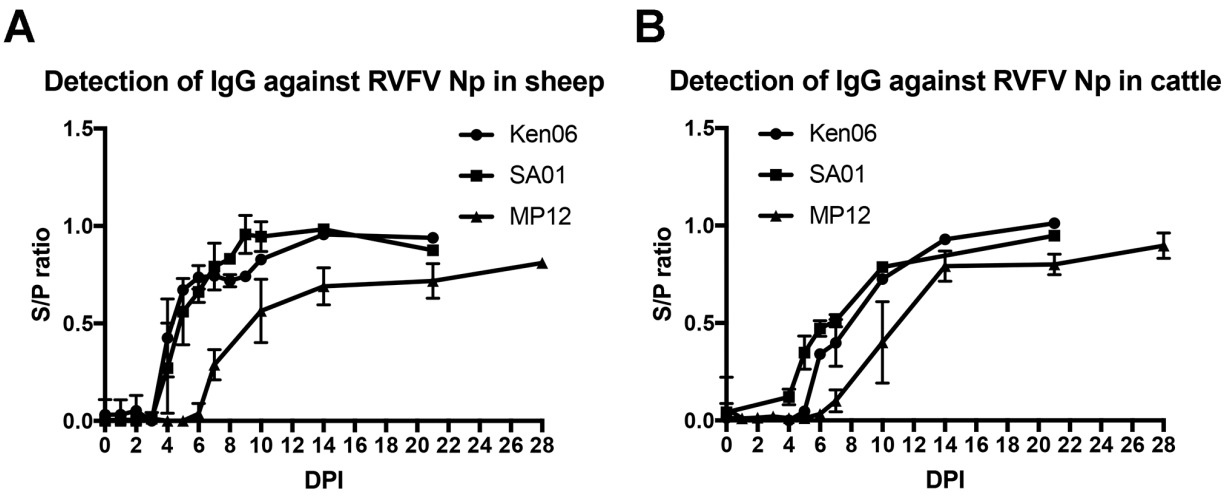

Figure 2.4 Detection of IgG antibodies in serum with RVFV N bead set during RVFV infections with SA01, Ken06, and MP12 strains.

Total IgG detection of all sheep and cattle are plotted by days post inoculation. (A) antibody detection in sheep; (B) antibody detection in cattle.

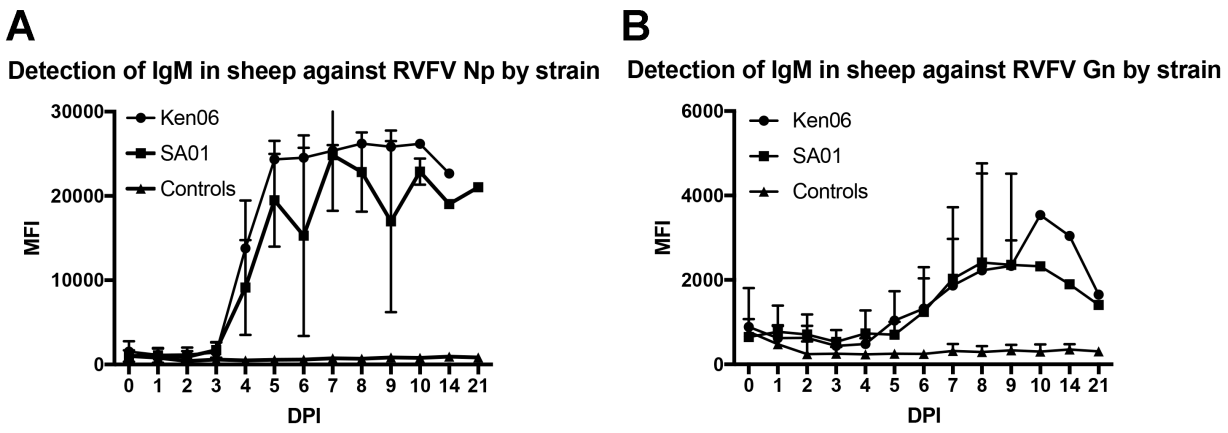

Figure 2.5 Detection of IgM antibodies in sheep serum over the course of experimental RVFV infections with SA01 and Ken06.

Total IgM detection of all sheep against the RVFV N target (A) and Gn target (B) are plotted by days post inoculation (DPI). The controls are serum samples from sheep that were mock-inoculated.

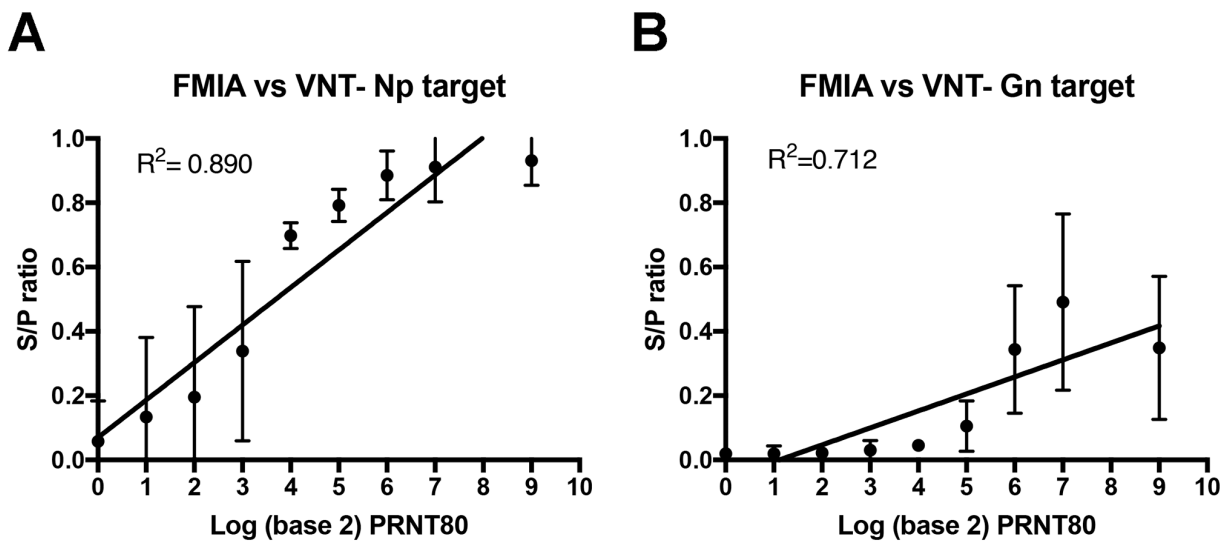

Figure 2.6 Comparing virus neutralization test (VNT) to FMIA.

FMIA results are represented on the *y* axis as an S/P ratio; VNT represented on the *x* axis as a log 2 of serum dilutions. Correlation coefficients (R^2 value) between VNT and FMIA were determined from the wild-type RVFV strains (Ken06, SA01). (A) correlation between the FMIA N target to VNT (P value <0.05); (B) correlation between the FMIA Gn target to VNT (*P* value <0.05).

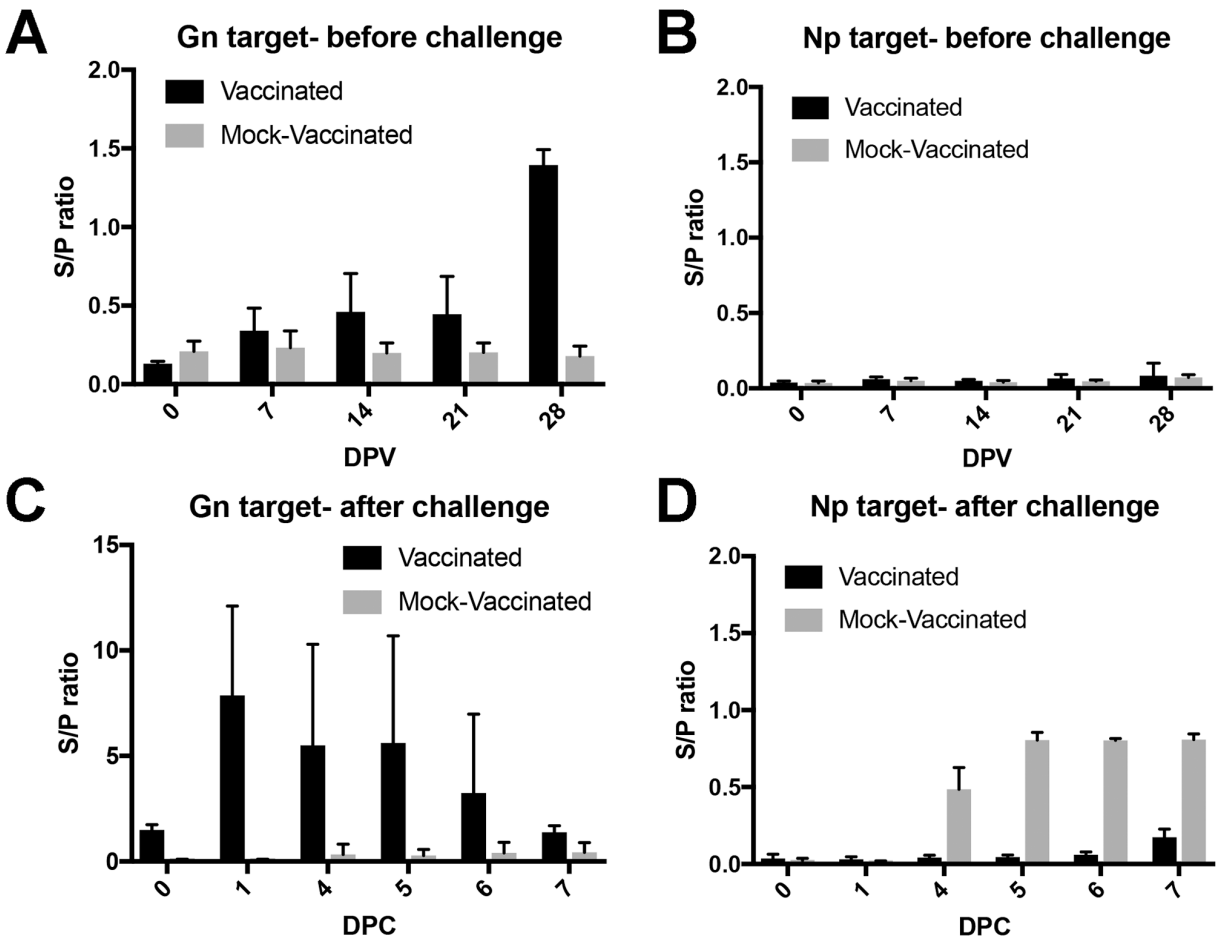

Figure 2.7 RVFV Np/Gn FMIA enables differentiation of vaccinated from infected sheep.

Sheep were initially vaccinated with a Gn/Gc subunit vaccine then challenged with Ken06 and maintained for 7 days. The subunit vaccine was given at 0 days post vaccination (DPV) then boostered at 21 DPV. Detection of IgG antibodies in sera against the Gn targets (A) and the N target (B) are shown. At 35 DPV all sheep were challenged (0 DPC). IgG production in sera was monitored using the Gn target (C) and the N target (D). N served as the DIVA-compatible marker.

Table 2.1 Experimental source and sample count for sera used in the evaluation of FMIA.

References of each study is included for additional details. DPI represents the last day sera was collected in the study. # of samples include total number of samples run on FMIA.

Study	Species	Age	Virus	Vaccinated?	DPI	# of samples
Wilson et al., 2014(97)	Sheep	4 months	MP12	No	28	94
	Cattle	4 months	MP12	No	28	94
Wilson et al., 2016(207)	Cattle	4-5 months	Saudi Arabia 2001-1322	No	21	26
			Kenya 2006-128b-15	No	21	23
			Mock inoculated	No	21	11
Faburay et al., 2016(206)	Sheep	4-5 months	Saudi Arabia 2001-1322	No	21	32
			Kenya 2006-128b-15	No	21	33
			Mock inoculated	No	21	20
Faburay et al., 2014(106)	Sheep	adult	None	Gn/Gc Subunit vaccine	49	48
Faburay et al., 2016(177)	Sheep	4-5 months	Kenya 2006-128b-15	Gn/Gc Subunit vaccine	7	45
Morrill et al., 2012(102)	Sheep	adult	None	arMP12-NSm delete 21/384	69	119
Weingartl et al., 2014(66)	Sheep	4 months	ZH501	No	35	4
Wilson, unpublished	Cattle	4 months	ZH501	No	35	7
RVFV- negative	Sheep	various	None	No	N/A	165
RVFV- negative	Cattle	various	None	No	N/A	325

Table 2.2 Diagnostic accuracy of RVFV FMIA for sheep and cattle serum samples

The disease status was categorized by VNT results. The cut-off values were determined by two-graph receiver operating characteristic (TG-ROC). CI, confidence interval; YI, Youden's index; AUC, area under the curve.

Sheep	Bead Set	Cutoff (MFI)	Sensitivity	Specificity	YI	AUC
TG-ROC Results	N	2500	94% (95% CI, 89 to 97%)	92% (95% CI, 88 to 95%)	85%	95%
	Gn	3800	89% (95% CI, 85 to 92%)	67% (95% CI, 59 to 75%)	56%	83%
Cattle	**Bead Set**	**Cutoff (MFI)**	**Sensitivity**	**Specificity**	**YI**	**AUC**
TG-ROC Results	N	13000	98% (95% CI, 97 to 99%)	97% (95% CI, 83 to 100%)	95%	99%
	Gn	9400	99% (95% CI, 97 to 99%)	51% (95% CI, 37 to 65%)	50%	74%
			Sensitivity	**Specificity**		
Series			83%	98%		
Parallel			99%	62%		

Chapter 3 - The Detection of RVFV by *In Situ* Hybridization

Preface

A portion of the results of this chapter will be submitted for peer review and publication under the title:

Differentiating Infected from Vaccinated Animals Compatible Detection of Rift Valley Fever Virus by *In Situ* Hybridization in Formalin-Fixed, Paraffin-Embedded Tissues

I.K. Ragan[1], W. C. Wilson[2], J.T. Trujillo[1], and A.S. Davis[1]

[1]Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State, Manhattan, KS, USA; [2] Arthropod-Borne Animal Diseases Research Unit, USDA, ARS, Manhattan, KS, USA

Introduction

In situ hybridization (ISH) assays have been used since 1968 (221) to evaluate gene expression, analyze cell proliferation, and detect infectious diseases (222). ISH originally was developed to detect and localize nucleic acid in cytological preparations and has since expanded to visual detection of nucleic acid in tissues. Early ISH assays detected DNA targets because of the high copy number present in cells compared to RNA. Optimizations of ISH techniques eventually enabled the detection of RNA (223), but these techniques required larger probe design, which made hybridization in tissues difficult, and assay sensitivity was poor if RNA was not present in high abundance (224, 225). Recent ISH technology advances provide rapid, robust, and highly accurate molecular platforms to analyze RNA, including viral RNA in diagnostic tissues. Currently, several companies offer unique detection systems and custom probe design capable of both singleplex and multiplex ISH detection. These new systems support more sensitive and rapid detection compared to traditional ISH approaches. Moreover, the ISH technology has also expanded from use restricted largely to cell preparations to diagnostic testing in formalin-fixed, paraffin-embedded (FFPE) tissues, which is extremely valuable for the testing high-consequence viral pathogens. First, we compare ISH to other molecular detection techniques in FFPE tissues. Second, we review some of the current ISH technologies that enhance RNA detection. Third, we discuss our work with two commercial options, Exiqon and RNAscope, to develop RVFV ISH in FFPE tissues. Finally, we present future directions for validating ISH for RVFV detection.

Molecular detection methods in tissues

Unlike blots and polymerase chain reaction (PCR), ISH provides sensitive detection of nucleic acids with cellular and tissue architecture context (226, 227). Additionally, where PCR requires RNA extraction for detection, current ISH techniques maximize the use of valuable tissues without further processing (228). Unlike immunohistochemistry (IHC), which detects protein expression, ISH detects nucleic acids and can differentiate virus types or strains in tissues (229, 230). Therefore, ISH is a valuable tool in research and diagnostic testing, a distinct alternative approach to molecular detection in tissues.

FFPE tissue sections are widely used in routine diagnostic procedures. They are a safe resource for handling infectious pathogens and cost-effective for long-term storage. Additionally these tissues are valuable resources for retrospective case studies (231). However, DNA and especially RNA can fragment before, during, and after formalin-fixation. Furthermore, formaldehyde induces nucleic acid-protein crosslinkages that decrease nucleic acid accessibility (232, 233). While, DNA ISH for FFPE tissues in a clinical setting is in regular use, the adoption of RNA ISH has been limited due to assay complexity and poor sensitivity (228). Recent advances in ISH technology have improved signal amplification and reduced the technical complexity of RNA detection in FFPE tissues. RNA ISH is now a valuable alternative to reverse transcription PCR (RT-PCR) for viral pathogen detection in FFPE tissues.

Traditional *in situ* hybridization detection methods

The first ISH methods used radiolabeled probes for detection (221). Radiolabeled ribonucleotides or deoxynucleotides are incorporated during cDNA synthesis or attached to the 3’ end of probes by terminal transferase respectively (234). Oligonucleotide probes are

commonly labeled with sulfur isotype ^{35}S, which is considered highly sensitive and provides high-resolution imaging. Other isotypes that are used include phosphorus (^{32}P, ^{33}P) and tritium (^{3}H) (222, 235). Nonetheless, radioactive probes pose health risks and require safe handling and complex disposal. Additionally, the required exposure time (up to 1-2 weeks), sensitivity decay during long-term storage, and the need for a dark room limits this method's use (222). Therefore, non-radioactive probes were developed for fluorescent detection followed by chromogenic detection (224). Most non-radioactive labels use haptens, like biotin or digoxigenin (DIG), that can subsequently be detected with antibodies conjugated to a detection molecule (236). DIG is reported to be more sensitive in detection compared to biotin due to non-specific binding of probes to endogenous biotin in tissues (222, 237). Using chromogenic detection provides a permanent signal, lower cost means of detection, and easy review of tissue context with use of a standard counterstain (238). Unlike chromogenic detection, fluorescent detection permits the direct visualization of targets and excellent localization of individual genes when multiplexing (239). However, fluorescent signal fades with long term storage even when slides are placed in cold storage and visualization requires a fluorescence microscope and filters (238). The first application of fluorescence ISH (FISH) incorporated fluorophore-labeled bases throughout the length of the probe (240). Since then FISH has evolved into many forms of direct and indirect detection and is well described (241). The use of non-radioactive probes for ISH has become the standard detection method as new probe designs improve the sensitivity and amplification of signal.

Current *in situ* hybridization detection methods

Fluorescence *in situ* hybridization

FISH has been used since the late 1970s (242) and is a rapidly growing detection method, especially for multiplex assays in live-cell imaging and gene analysis (224). More importantly, FISH is a popular technique for visualizing mRNA in FFPE tissues. Formally known as single molecule FISH, the technique has since been commercialized by companies like Stellaris® (Biosearch Technologies, Petaluma, CA), who design custom probes tailored to specific research needs. Custom probes are labeled on the 3' end with various fluorophores enabling the analysis of multiple transcripts simultaneously (243). Moreover, the ISH procedure is completed in one day and can be automated offering a rapid detection system in tissues (222). Commercial kits using FISH are available for rapid detection of individual pathogens. ID-FISH (ID-FISH Technology Inc., Palo Alto, CA) has designed kits to detect malaria and tuberculosis in blood or sputum, and is currently developing *Babesia, Bartonella*, and *Plasmodium spp.* FISH kits. Moreover, the kits are designed for field application in endemic countries where access to appropriate lab equipment may be limited (244). FISH has proven to be a versatile platform that is used for clinical testing of infectious diseases.

Rolling-circle amplification

Despite the popularity of FISH, there are limitations to the method including background from non-specific binding of fluorescent antibodies to cells and proteins (245). Careful selection of fluorophores and signal amplification methods is required to improve the signal-to-noise ratio, especially when signal intensity is weak due to low RNA copy number (246). An alternative method to achieve highly specific signal to single-molecule resolution is rolling-circle

amplification (RCA) (247). RCA can amplify signal rapidly by providing multiple sites for the annealing of labeled probes. Briefly, ISH probes bind to the target sequence then ligate into a circle by DNA ligase to create a circular template. MicroRNA acts as a primer to elongate the sequence and generate a continuous complementary strand that enables the annealing of multiple detection probes (248). The RCA technique is reported to have low efficiency (247) but with optimization, it is valuable in detecting low levels of RNA (249) and can be applied in a multiplex format (250) providing a clear signal with high sensitivity.

Branched DNA probes

One of the newest advances in ISH RNA detection is the use of branched DNA probes. There are currently two commercial branched DNA probe detection systems, RNAscope (Advanced Cell Diagnostics, Hayward, CA), and ViewRNA (Affymetrix, Santa Clara, CA). The technologies support both chromogenic and fluorescence-based detection in tissues but differ in their probe design. RNAscope uses a patented paired z probe design where both z probes (18-25 base pairs (bp) each) must hybridize contiguously on the target sequence to form a 28-base hybridization site for a preamplifier (Fig. 3.4). Requiring the paired probes to sit contiguously on the target sequence minimizes nonspecific hybridization of individual probes. The preamplifier contains several sites for amplifiers, which, in turn, contain multiple binding sites for modified label probes. The sequential hybridization of preamplifier, amplifier, and label probes enhances the signal for detection. A single signal dot represents the detection of one target molecule (228). Therefore, gene expression quantification is possible. ViewRNA technology combines several synthetic probes that each contain target-specific and blocking oligonucleotides to reduce non-specific binding. Individual oligonucleotide probes contain a lower region complementary to the

target RNA as well as an upper region to promote the branched-DNA signal amplification. The branched-DNA probes can detect viral RNA targets as small as 100 bp (251). ViewRNA can be used in clinical detection of weak positive samples such as rare hepatitis C virus cases (252). RNAscope offers automation for RNA detection, which provided consistency and reproducibility (253). Branched DNA probes represent a major advancement in the detection of RNA in cells and tissues.

Locked nucleic acid probes

Lastly, locked nucleic acid probes (LNAs) are newly designed probes that also have enhanced hybridization efficiency to RNA targets. LNAs are high-affinity RNA analogues wherein the ribose ring is "locked" in position to improve thermal stability during hybridization to a complementary RNA or DNA strand. The thermal stability enables normalization of probe melting temperature and the use of shorter length probes (13-20 bp) compared to traditional probes, which supports the detection of small targets like microRNA and single nucleotide polymorphisms (254, 255). This in turn improves probe accessibility in the tissue and improves binding affinity resulting in high sensitivity detection. Exiqon (Qiagen, Germantown, MD) uses LNA technology in their custom design of probes for RNA ISH detection in FFPE tissues. The LNA probe is labeled with DIG for chromogenic or with fluorescein for fluorescent detection. LNA probes are valuable in detecting single, short nucleic acid sequences such as microRNA in cells and tissues (248). However, target selection for FFPE RNA ISH will be challenging considering the aforementioned deleterious nucleic acid modifications that occur with FFPE tissue processing.

RVFV detection with *in situ* hybridization

Tissue samples are important in RVFV detection for they are typically the first sample type acquired and submitted to a laboratory during an outbreak (256). Infected tissues must be handled with care due to their zoonotic risk. Therefore, FFPE tissues offer a safe method for handling and testing of Select Agents such as RVFV (257). PCR, IHC, and ISH are routinely used for pathogen detection in FFPE tissues, however, validated platforms for RVFV detection are limited. The only reported use of ISH for RVFV research is single molecule vRNA fluorescent ISH to detect RVFV genes in fixed cells (160). There is no report of RVFV ISH for FFPE tissues. Furthermore, an ISH assay that can differentiate infected from vaccinated animals (DIVA) for RVFV or any other virus has not been previously reported. ISH can be an important diagnostic tool that provides a safe means to detect RVFV nucleic acids within the context of the tissue morphology.

We have recently applied two commercial technologies to the detection of RVF viral RNA in FFPE tissue: Exiqon and RNAscope. The advantages of these commercial platforms include consistent production of probes, assay repeatability, and simplified ISH protocol. First, we will describe ongoing work to develop an ISH assay to detect RVFV using Exiqon's technology. Then, we provide details on the successful RVFV detection with RNAscope technology. Lastly, we describe future directions for validating the RNAscope ISH assay.

Exiqon *in situ* hybridization for RVFV detection

Exiqon ISH offers custom ISH probes that are DIG labeled for chromogenic detection in FFPE tissues. Exiqon may be a better choice for endemic countries since probes can be lyophilized, which permits for stable handling and storage of reagents. Additionally, the assay

can be performed in one day and, unlike RNAscope, requires less specialized equipment and proprietary reagents. Therefore, there is a greater possibility to phase out the commercial reagents and use more affordable options. Our objective is to develop a high sensitivity ISH assay for RVF RNA detection in FFPE for use in endemic and non-endemic areas. This project is ongoing.

Materials and Methods

To date, testing of Exiqon ISH probes has been on RVFV Kenya-128B-15 (Ken06) strain infected sheep tissue from a prior challenge study (206). The uninfected control tissues were provided by the Kansas State University Veterinary Diagnostic Lab (KSU VDL). Two LNA probes targeting RVFV's M segment, one in the viral-sense and the other in the viral-antisense orientation, were synthesized by Exiqon using the consensus sequence of eight RVFV strains (RVF-Moz-Gob-T9-2014, RVF-Moz-Gob-T7-2014, Kenya-128B-15, SA01-1322, ZH-548, S Africa 1224_10, S Africa 85_10, S Africa 184_10). The Exiqon probe sequences are: antisense /5DigN/TCGCAGACCCCTTTCATTTTTGT/3Dig_N/ (bp 194-216), and sense /5DigN/CAACTCAGCACTGCACATGAGG/3Dig_N/ (bp 251-272). Additionally, a positive control probe was synthesized in antisense orientation to target the β actin housekeeping gene at bp 2042-2062 (probe sequence /5DigN/AGCCAGGTCCAGACGCAGGAT/3Dig_N/). The probe targets a consensus of human, cattle, goat, sheep, and mouse species sequences. Exiqon also provided a negative control probe (scrambled) (probe sequence /5DigN/GTGTAACAGTCTATACGCCCCA/3Dig_N).

ISH was performed using the miRCURY LNA miRNA ISH Optimization Kit for FFPE tissues (Qiagen; Germantown, MD). Briefly, 4 μm tissue sections were cut and placed on

positively-charged slides by KSU VDL Histology lab. The tissues were deparaffinized in xylene and rehydrated through graded alcohols (100%-70%) into PBS. Proteinase K diluted in Proteinase K buffer or steam retrieval using DAKO citrate pH 6 target retrieval solution (Aligent; Santa Clara, CA) was used for antigen retrieval prior to hybridization. Hybridization of probes was done according to manufacturer's instructions. The hybridization temperature was set for 30˚C below the RNA melting temperature of each probe. After hybridization the tissues went through a series of stringent graded washes with saline-sodium citrate (SCC) buffer (5x-0.2x), were blocked using 2% goat serum in PBS with 0.1% Tween (PBS-T), then incubated with an anti-DIG primary antibody conjugated with alkaline phosphatase (AP) or horseradish peroxidase (HRP) to detect the probes (Sigma Aldrich; St. Louis, MO). For AP detection, BCIP/NBT AP substrate was applied according to manufacturer's instructions (Vector Laboratories; Burlingame, CA). For HRP detection, 3,3'-diaminobenzidine (DAB) substrate was applied according to manufacturer's instructions (Vector Laboratories; Burlingame, CA). The tissues were lightly counter-stained with Nuclear Fast Red (Vector Laboratories; Burlingame, CA), rinsed in tap water, and dehydrated using graded alcohols (70%-100%) and xylene. The slides were mounted with Permount Mounting Medium (Electron Microscopy Sciences; Hatfield, PA) and reviewed as described prior (206).

Results

Preliminary experiments were completed using either the RVFV probes or the positive control probe in conjunction with the negative control probe. Optimization of the Exiqon ISH has focused on enzymatic versus heat antigen retrieval, length of time for antigen retrieval, hybridization time, change in hybridization oven to increase precision, and AP versus HRP-

based detection systems. The first experiment tested control probes using an AP detection system. Yet no signal was detected. Next, the hybridization time was extended from 60-120 minutes (min). The third run included testing of the two RVFV ISH probes against the M gene using RVFV infected tissue. No signal was detected from the two RVFV probes, therefore, further testing focused on optimizing the assay using only the control probes. The control probes were tested again with various concentrations of Proteinase K and increasing the length of time for antigen retrieval. Next, a steam antigen retrieval system was tested as well as HRP and AP detection systems were compared. There was still no signal despite optimization of the critical steps in ISH. To eliminate the risk of poor handling and storage of probes at time of shipment, a new β actin probe was ordered. The new probe was tested using both steam and enzymatic retrieval. Additionally, the probe was tested with HRP and AP detection systems. The new probe did not show signal. To ensure the AP labeled anti-DIG antibody was detecting our ISH probes, a new antibody was ordered as per vendor's recommendation. The last experiment to date included using a new hybridization oven to ensure the temperature remained constant during hybridization. No specific signal has been detected from the experiments thus far.

Future Directions for RVFV Exiqon *in situ* hybridization

Future experiments include testing sensitivity of probes by dot blotting. Additionally, we will test the AP and HRP detection system and ensure the primary antibody's ability to detect the double-DIG labels on the custom LNA probes. Once we demonstrate the sensitivity of the probes, all reagents and controls need to be optimized. Optimization of the new β actin probe has yet to be done. This includes decreasing the hybridization temperature to decrease hybridization stringency and testing various Proteinase K concentrations to improve penetration of probes in

FFPE tissues. Once successful, the Exiqon ISH can offer a flexible and affordable means of testing RVFV in tissues.

RNAscope *in situ* hybridization for RVFV detection

RNAscope has been successfully demonstrated for detecting viral RNA in FFPE tissues for many viral pathogens (258-262). To our knowledge this technology is untested for RVFV detection. After the technical issues with Exiqon we tried RNAscope ISH. It was not our first choice due to its higher price point.

Several DIVA-compatible RVFV modified-live vaccines are currently in development for livestock. Large deletions in NSm and NSs genes improve the safety of modified-live vaccines like MP12 (102). There are three NSs deletion vaccine (ΔNSs) strains, including Clone 13 (90, 91), the recombinant MP12 (103, 104) and ZH501 strains (17). For NSm deletion vaccine (ΔNSm) strains, there is the recombinant MP12 strain (100, 101) and the recombinant ZH501 strain (17). Finally, there is the ZH501strain ΔNSm/ΔNSs (16). The consensus deletion for the ΔNSs modified-live vaccines (MLVs) is ΔNSs81/191 in the S segment and the consensus deletion for the ΔNSm MLVs is ΔNSm22/384 in the M segment. If any of these MLVs were to be used prophylactically or in response to an outbreak in a non-endemic region, tools to support assessment of return to 'disease free' status would be critical.

There is a need for DIVA-compatible molecular assays with these MLVs. Currently, the only such validated tool is the NSs exclusionary multiplex quantitative reverse transcription PCR (RT-qPCR assay) (147). The multiplex RT-qPCR for the ΔNSs strains was evaluated with infected cells and sera, allowing for early RVFV detection. No efficacious DIVA assay for the

ΔNSm strains currently exists. Therefore, a validated molecular DIVA assay for the ΔNSm and ΔNSs vaccine strains is of high priority.

The objectives of the RNAscope ISH development were two-fold: (1) to develop a highly sensitive ISH assay for the detection of RVFV RNA in FFPE tissue using RNAscope and (2) to develop duplex DIVA ISH assays, capable of differentiating ΔNSm and ΔNSm MLVs from a virulent RVFV strain.

Materials and Methods

Tissues

All tissues are summarized in Table 3.1. RVFV-infected mouse tissues came from 2 studies in which inoculation was: (1) subcutaneously with $7.9x10^3$ pfu of Ken06 wildtype strain (263) and (2) intraperitoneal with $1.0x10^3$ pfu of ZH501-ΔNSm21/384 or ZH501-ΔNSs16/198 vaccine strain (17). The infected ruminant tissues came from studies in which the sheep and cattle were respectively inoculated with $1\text{-}2x10^6$ pfu of the Ken06 strain (206, 207). Uninfected control tissues came from either mock-inoculated animals in the aforementioned studies or were species matched KSU VDL tissues.

ISH probes design

The pan-RVFV and two DIVA exclusionary probes were synthesized by Advanced Cell Diagnostics (ACD; Hayward, CA) according to investigator provided target sequences. Seven RVFV sequences representative of phylogenetic variance were aligned (MP12, Ken06, Clone 13, ZH548, ZH501, Smithburn, and Saudi Arabia 2001-1322). The pan-RVFV probe consisted of 20 z pairs targeting the RVFV L segment consensus sequence between nucleotides 2-801. The

DIVA exclusionary NSm probe, M DIVA probe, consisted of 8 z pairs targeting the RVFV ΔNSm deletion sequence between nucleotides 3-392. The DIVA exclusionary NSs probe, S DIVA probe, consisted of 2 z pairs targeting the RVFV consensus S segment ΔNSs between nucleotides 81-191. For the pan-RVFV ISH assay, the pan-RVFV probe was tested in singleplex. The two DIVA duplex assays used one of the DIVA probes in conjunction with the pan-RVFV probe. ACD recommended negative and species-specific positive control probes were also used.

RNAscope ISH

The singleplex and duplex chromogenic ISH assays were conducted according to manufacturer's protocol. The duplex assay was performed using the custom RVFV probes and the RNAscope 2.5 HD Duplex Kit (ACD; Hayward, CA). The singleplex assay was performed using the custom RVFV L probe and the RNAscope 2.5 HD Brown Kit (ACD; Hayward, CA). Briefly, FFPE tissues were sectioned at 4 μm, mounted on positively charged slides, and air dried overnight. The slides were baked for 1 hour (hr) at 65˚C, deparaffinized in xylenes, and rehydrated in 100% alcohol. Preparation and pretreatment of tissues followed the manufacturer's protocol for FFPE samples using the standard pretreatment times of 15 min for all slides. Probes were added to tissues and incubated for 2 hr at 40˚C in a HybEZ oven (ACD; Hayward, CA). Following hybridization, the slides were washed with 1x Wash buffer and stored in 5x SCC (Thermo Fisher Scientific; Waltham, MA) overnight at RT. Amplification of hybridized probes followed the manufacture's protocol for the Duplex (Chromogenic) Detection Kit and the Singleplex Brown Detection Kit. Slides were counter stained with 100 % Mayer's hematoxylin solution (Electron Microscopy Sciences; Hatfield, PA) and briefly rinsed in tap water as a bluing reagent. To mount slides for the duplex assay, slides were dried for 15 min at 60˚C and mounted

using Vectamount permanent mounting medium (Vector Laboratories; Burlingame, CA). To mount slides for the singleplex assay, slides were dehydrated according to the manufacturer's protocol and mounted with Permount mounting medium (Electron Microscopy Sciences; Hatfield, PA). Slides were reviewed and images captured with a DP27 camera (Olympus; Center Valley, PA) on a BX46 light microscope using CellSens Standard version 1.16.

Reverse transcription polymerase chain reaction

All ruminant FFPE tissues were previously tested by RT-qPCR (Upreti, et al., unpublished). The mouse ΔNSm and ΔNSs FFPE tissues were tested using the same methods. Briefly, five 4 µm thick FFPE tissue sections per sample were treated with deparaffinization solution (Qiagen; Germantown, MD), lysed with ATL buffer and proteinase K then RLT buffer (Qiagen; Germantown, MD), total RNA extracted using magnetic bead extraction (Genereach; Taichung City, Taiwan) on the Taco^{TM} mini Automatic Nucleic Acid Extraction System (Genereach; Taichung City, Taiwan) and RT-qPCR performed using Quanta qScript XLT 1-Step RT-qPCR ToughMix (Quanta BioSciences; Inc, Beverly, MA) on the Bio-Rad CFX thermocycler (Bio-Rad, Hercules, CA). The optimized cycling conditions were 50˚C for 20 min, 95˚C for 10 seconds (sec), 60˚C for 1 min followed by 45 cycles of 95˚C for 10 sec. The cut-off cycle threshold (Ct) value was set at 38.

Immunohistochemistry

IHC results for all Ken06 ruminant tissue slides were previously published (206, 207). Mouse tissue IHC was done according to published methods (264) with an adjusted primary

antibody dilution of 1:8000. Finally, for comparison of IHC and ISH results within lesion context, hematoxylin and eosin slides were made by KSU VDL following standard procedures.

Results

The pan-RVFV singleplex assay readily identified RVFV RNA in lesions of multiple tissue types from both sheep (Fig. 3.1 A-B) and cattle (Fig. 3.1 C). It did not label uninfected tissues (Fig. 3.1 D). The negative and positive control probes performed as expected (Fig. 3.2).

The M duplex assay differentiated Ken06 infected mouse liver (Fig. 3.3 A) from ΔNSm infected mouse liver (Fig. 3.3 B). Both probes, pan-RVFV and M DIVA, labeled the wildtype infected tissues while the pan-RVFV probe only labeled the ΔNSm infected tissues. Similar results were seen with infected mouse spleen (data not shown). Likewise, the S duplex assay similarly differentiated Ken06 infected mouse brain (Fig. 3.3 C) from ΔNSs infected mouse brain (Fig. 3.3 D) and liver (data not shown).

The ISH results on FFPE tissues correlated 100% with previously tested RT-qPCR results (Table 3.1). All positive tissues tested had low Ct values ranging from 21.3-23.6, which correlated well with a strong signal by ISH. As expected the ΔNSs tissue's RT-qPCR was negative for the S segment. ISH labeling correlated with histopathology lesions and with IHC. On average, ISH produced a stronger, more widespread and more readily interpreted signal (Fig. 3.4).

Discussion

Here we demonstrated not only RNA ISH to detect RVFV RNA in FFPE tissues but also DIVA ISH to differentiate RVFV ΔNSs and ΔNSm MLVs RNA from that of RVFV wildtype

Ken06. This is the first report of RVFV ISH assays that are appropriate for diagnostic use. Additionally, we demonstrated the use of ISH to differentiate vaccine from virulent wild type virus strains in FFPE tissue blocks for RVFV.

RNAscope® ISH technology is a highly sensitive RNA ISH platform previously shown to accurately detect viral nucleic acids in FFPE tissues, including those of porcine parainfluenza virus 1 (265), simian immunodeficiency virus (260), human immunodeficiency virus (259, 266), equine arteritis virus (267), and papillomaviruses (268-270).We found that the pan-RVFV probe readily identified vRNA regardless of infecting strain in all species' tissues tested (Fig. 3.1 and 3.3). Additionally, the ISH results correlated well with results from IHC and RT-qPCR for the same FFPE tissue blocks (Table 3.1 and Fig. 3.4).

Both of the M and S duplex assays readily differentiated the respective ZH501 deletion vaccine strains from the wildtype Ken06 strain (Fig. 3.3). The M duplex assay, based on the pan-RVFV probe in combination with the M DIVA probe targeting the ΔNSm MLV's deletion sequence in RVFV's M segment, differentiated ZH501-ΔNSm21/384 from Ken06 in FFPE mouse liver and spleen. Based on the design of the M DIVA probe, the M duplex assay should also be compatible with other candidate ΔNSm strains (101, 102). Surprisingly, the S duplex assay readily differentiated ZH501-ΔNSs16/198 from Ken06 in mouse brain despite only having 2 z pairs to detect the deletion sequence. ACD recommends that RNAscope probe design should use 3-6 z pairs minimum per probe for adequate signal detection. With the S duplex assay targeting the consensus deletion at 81-191 on the S segment, the ISH probe should be compatible with all ΔNSs strains (16, 91, 102-104, 271).

The advantages to using RNAscope® technology include a low signal to noise ratio and high sensitivity due to probe design. The probes have a unique double z design allowing for

amplification of target-specific signal while maintaining a low background noise by reducing non-specific hybridization of the probes (Fig. 3.5). Each pair of z probes must hybridize contiguously to the target sequence in order to form a 28-base pair hybridization site for a preamplifier. Then, through a series of amplifiers and labeled probes that attaches to the preamplifier, increases the assay's detection power (228). This assay can be automated to improve repeatability and shorten assay time.

The high costs may limit the application of the RNAscope® pan-RVFV ISH assay as a routine diagnostic test. Yet, it has great potential as a confirmatory diagnostic test or RVFV pathogenesis research tool including for use with archival FFPE samples. Moreover, the DIVA duplex assays are suitable for use during an outbreak wherein the response includes vaccination with an RVFV ΔNSs and/or ΔNSm strain.

The results demonstrate that RNAscope® is a sensitive ISH platform that can detect RVFV RNA in FFPE tissues. This work can be conducted outside high containment once formalin-fixation inactivation of the virus has been confirmed. More importantly, RNAscope® ISH assays can be DIVA compatible enabling differentiation of RVFV deletion vaccine from wildtype strains. This novel concept is extendable to the management of other transboundary animal diseases employing MLVs as well as viral vector-based vaccines in their prevention and control strategies.

Future Directions for RVFV RNAscope ISH

With successful detection of RVFV RNA using the RNAscope ISH, further validation of the assay is needed to determine diagnostic accuracy. The first step is to optimize all reagents and controls in the RNAscope assay. Second, additional experimental tissues and field samples

need to be tested with the pan-RVFV ISH then compared to RT-qPCR and IHC to determine diagnostic sensitivity and specificity. Third, we are interested in testing the DIVA capability of the RVFV ISH to detect additional gene deletion recombinant vaccines using the RNAscope duplex assay. Fourth, we want to investigate means to semi-quantify and quantify ISH results, which will permit the correlation to IHC and RT-qPCR, respectively. Finally, we want to develop a dual ISH-IHC procedure to use in RVFV pathology studies. The intent of these experiments is to move the ISH assay beyond proof-of concept and to validate the assay for routine use in diagnostics and research.

To proceed with validating the RNAscope ISH assay all reagents and controls need optimization. This includes running additional samples to demonstrate robustness and repeatability of the assay. Analytical specificity of ISH probes should be tested on non-RVFV infected tissues such as Schmallenburg virus or Cache Valley virus, both members of the order *Bunyavirales*. Additionally, it is of interest to test the RVFV probes on goat and other African ungulate tissues to demonstrate the versatility of the assay with tissues from various ruminant species. This would be of great value when using the assay in endemic countries where these ruminants are important reservoirs for RVFV.

Additional experimental tissue samples from RVFV sheep and cattle challenge studies will be tested using the pan-RVFV ISH assay (206, 207). The experimental tissues have previously been tested by IHC and RT-qPCR (272), which can be used to establish diagnostic accuracy estimates for ISH by using a latent class analysis. Furthermore, the RNAscope assay will be applied to naturally infected FFPE tissues from the 2010 South African outbreak through a research collaboration with the University of Pretoria, South Africa to evaluate the accuracy of the assay on field samples. These samples will be tested by RT-qPCR and ISH in parallel.

It is important to evaluate the ISH probes' ability to detect a diversity of RVFV strains. To expand the number of strains detected by ISH additional vaccine strains as well as naturally infected samples will be tested. In particular, testing the DIVA duplex assay with dual ΔNSm-ΔNSs deletion vaccine infected tissues is of interest. A study to acquire hamster tissues infected with MP12-ΔNSm/ΔNSs deletion vaccine is planned. Finally, there is interest in an ISH assay that can detect single nucleotide polymorphisms within the RVFV MP12 strain, which contains point mutations spread across all three gene segments (273).

To compare ISH to IHC and RT-qPCR, the ISH signal will require interpretation as semi-quantitative and quantitative results, respectively. Detection by RNAscope ISH can be correlated to IHC using a semi-quantitative score comparison. Typically, the number of positive cells per 10 high-magnification (400x) fields is determined and a sample score assigned accordingly (267, 268). An example scoring scale would be 0= no signal, 1= ≤ 10 positive cells, 2= 11-30 positive cells, 3= 31-50 positive cells, 4= 51-75 positive cells, 5= >75 positive cells. To compare RNAscope signal to RT-qPCR, the number of individual signals needs to be counted. Wang, et al. suggests individual dots represent single molecules of viral RNA (228). Therefore, by using an image analysis software to count individual signal dots, the quantitative results can be correlated to copy number per cell. Recently, a custom-designed algorithm with the image analysis software, Definiens (Definiens AG, Munich, Germany) counted RNAscope signal from HER2 mRNA in breast carcinomas, then compared to copy number per cell determined by RT-PCR (274). Halo software (Indica Labs, Corrales, NM) is another image analysis software that can quantify ISH signal (253). A Spearman correlation can be used to correlate RNAscope dot signal to RT-qPCR copy number.

Lastly, ISH and IHC are valuable tools for investigating RVFV expression on both the mRNA and protein expression levels, respectively. Combining ISH with IHC provides the correlation of mRNA transcripts with post-translational control for target genes (275). Localizing mRNA and protein expression to individual cells with high sensitivity using one slide is advantageous for precious samples. A dual ISH-IHC assay using RNAscope was successfully demonstrated in mouse retina FFPE tissues and free-floating brain sections (275, 276). We propose developing dual ISH-IHC assays to provide simultaneous analysis of RVFV mRNA and protein antigens. The pan-RVFV singleplex ISH assay will be applied to infected tissues followed by detection with IHC as described in Chapter 4. This dual assay provides a tool for understanding RVFV pathology at the level of both protein and gene expression within infected FFPE tissues. Moreover, this assay can be applied to archival samples where availability of fresh tissue is limited due to Select Agent regulations or for retrospective studies of previous RVFV outbreaks.

Conclusion

With the recent advances in probe technology and improved detection systems, ISH is a powerful tool for detecting viral pathogens in tissues. Currently, there is no ISH for the detection of RVF viral RNA in FFPE tissues. We recently evaluated two commercial technologies, Exiqon and RNAscope, for the detection of RVFV. While, to date, we have been unsuccessful with the Exiqon ISH approach, our results with RNAscope are promising. Future directions with the RNAscope assays include determining pan-RVFV probe diagnostic accuracy, testing it with other RVFV strains, benchmarking ISH against IHC and RT-PCR, and developing a dual ISH-IHC assay to detect RVFV RNA and proteins together. The pan-RVFV ISH is a valuable

diagnostic assay and research tool. Continuing work on lowering costs of these assays using in-house reagents will promote their wider use, especially in endemic countries. Most importantly, the duplex ISH assay is compatible with RVFV candidate deletion vaccines and can be used as a surveillance tool to control the spread of RVFV.

Acknowledgments

The authors thank Juergen Richt (Kansas State University) and Tetsuro Ikegami (University of Texas Medical Branch) for access to formalin-fixed, paraffin-embedded tissue blocks. The authors also thank Deepa Upreti for technical assistance with RT-qPCR and the staff of the KSU VDL Histopathology Unit. This work was funded by the USDA Agricultural Research project number 3020-32000-009-00D, and the Department of Diagnostic Medicine, College of Veterinary Medicine, Kansas State University's startup funds for Dr. Davis. No competing financial interests exist.

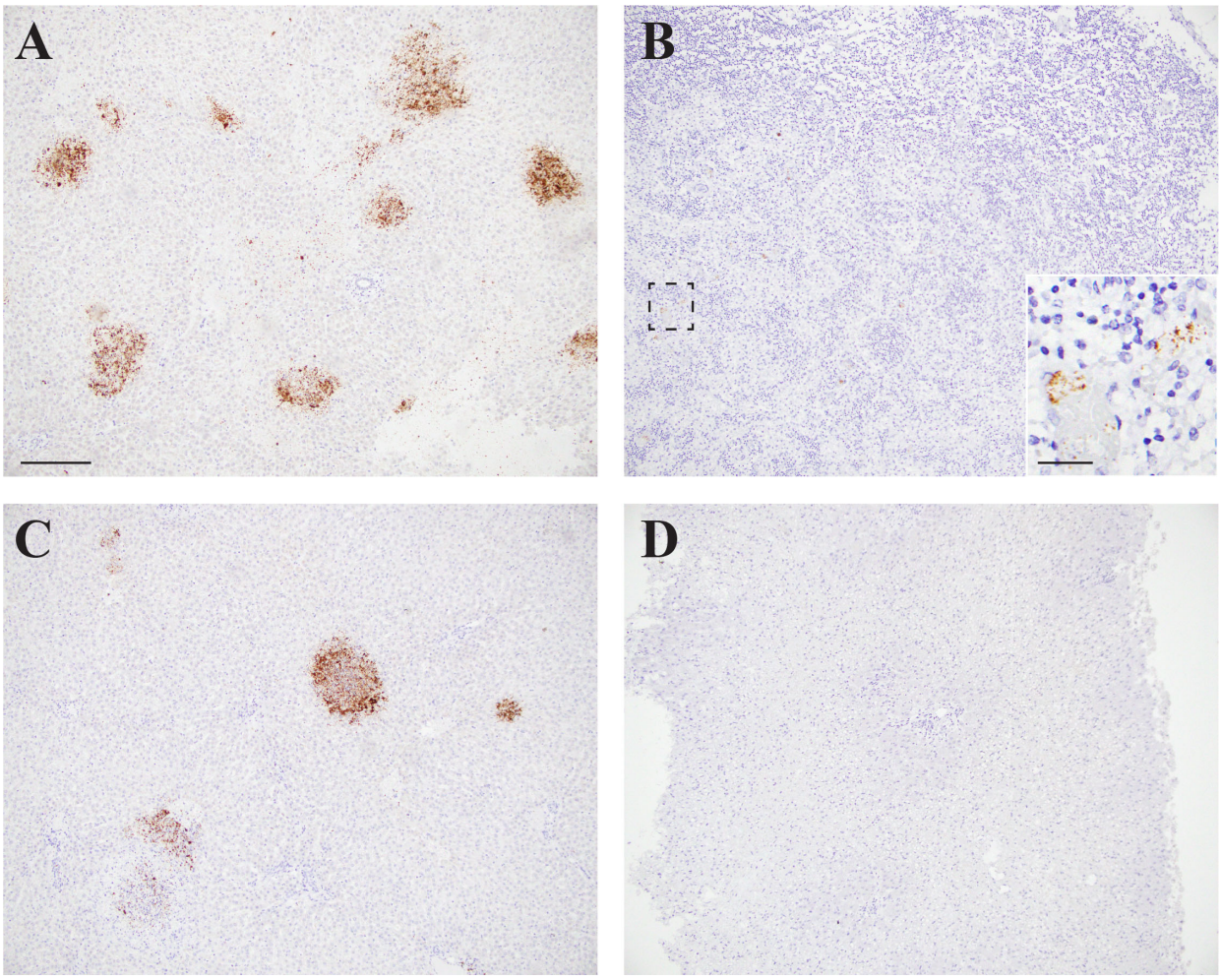

Figure 3.1. Pan-RVFV ISH detects Ken06 in both sheep and cattle tissues

The pan-RVFV probe labeled RVFV RNA (brown) in (A) Ken06 infection induced hepatic necrosis lesions in sheep; (B) shows that the probe labeled the cytoplasm of macrophages circulating in the medullary sinuses of an infected mesenteric lymph node, inset is a magnification (mag) of the region outlined by the dashed box. Labeling of Ken06 lesions in cattle liver was similar to sheep (C). The signal was punctate and cytoplasmic consistent with RVFV intracytoplasmic replication. No signal was present in the uninfected control tissues, (D), sheep liver. The bar for the main images is 200 µm and for the insets is 25 µm.

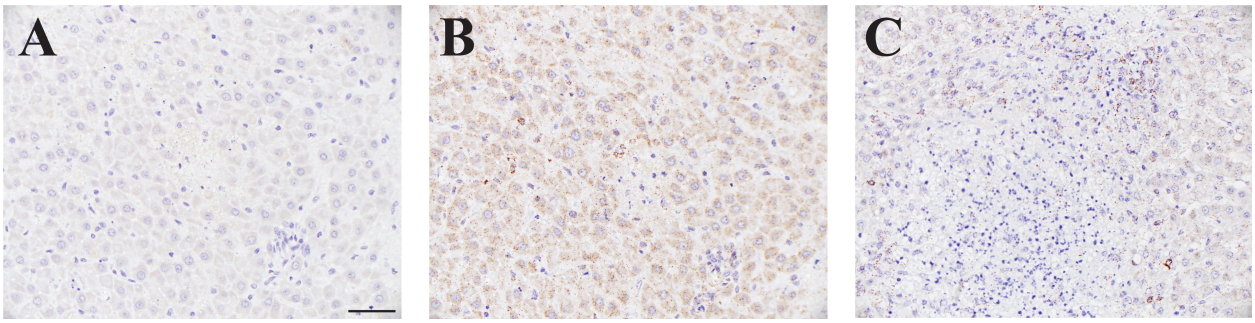

Figure 3.2 RNAscope positive and negative control probes tested on ruminant FFPE tissues

A universal negative control probe against the DapB gene of *Bacillus subtilis* used on RVFV-infected sheep liver (A) shows no signal. A positive control probe against the PPIB gene was used on RVFV-infected sheep liver (B) and on RVFV-infected cattle liver (C) to assess tissue RNA integrity and assay procedure. The positive control probe shows punctate labeling in the cytoplasm of intact cells. The bar for the images is 50 µm.

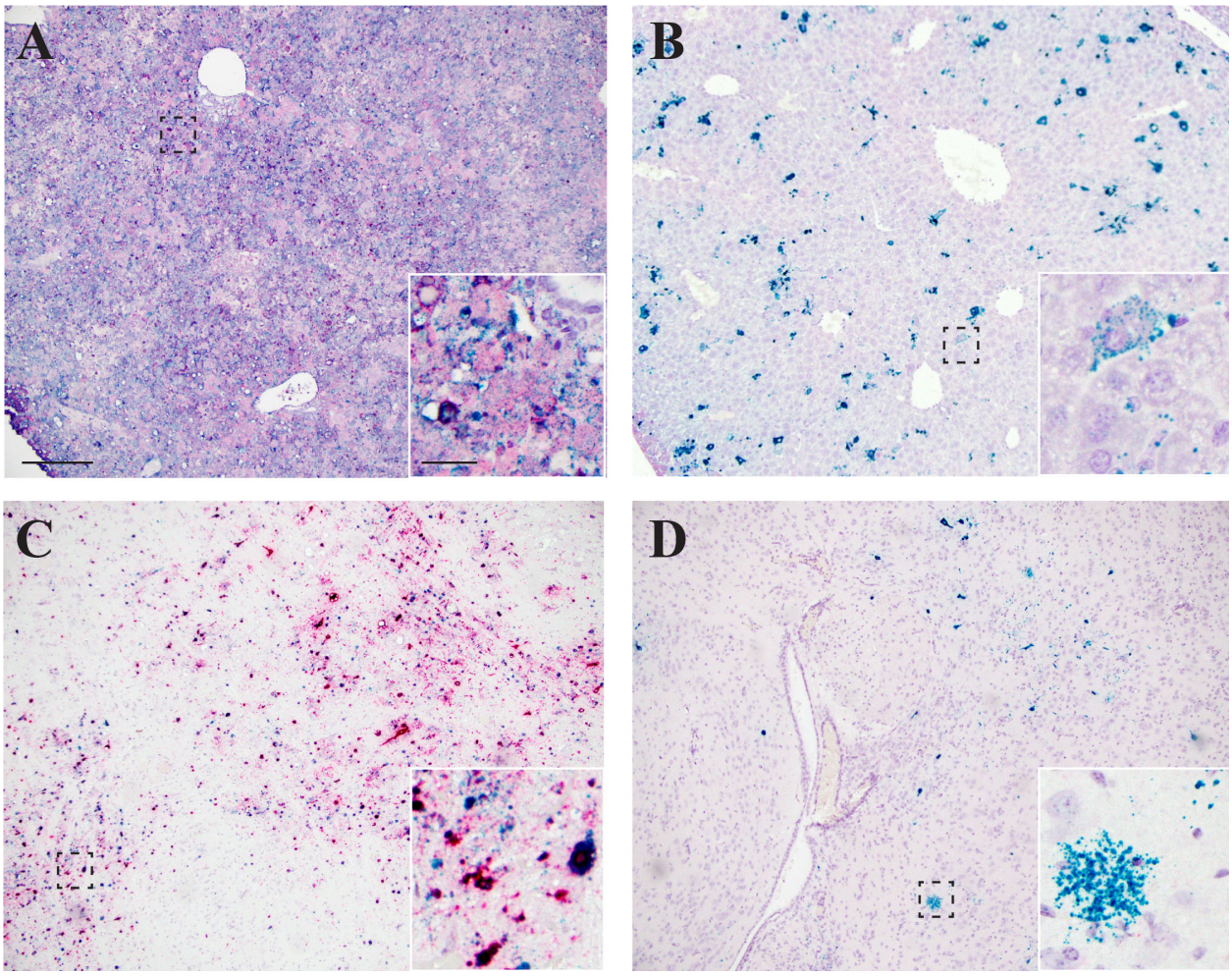

Figure 3.3 The DIVA ISH duplex assays differentiate ΔNSm and ΔNSs from Ken06

RVFV M DIVA and S DIVA probes were each used in duplex with pan-RVFV probe. (A) Both the pan-RVFV probe (green) and M DIVA probe (red) strongly labeled RVFV RNA in Ken06 infected mouse liver. The inset is a magnification (mag) of the region outlined by the dashed box and shows cytoplasmic dual labeling of infected hepatocytes that is punctate to coalescing due to heavy viral loads. (B) Only the pan-RVFV probe labeled RVFV RNA in ΔNSm infected mouse liver. The inset is a mag showing a hepatocyte with a moderate degree of intracytoplasmic punctate labeling. Scattered signal is present within adjacent cells. There is no M DIVA probe signal. (C) Both the pan-RVFV probe (green) and the S DIVA probe (red) labeled Ken06

infected mouse brain. The inset is a mag showing the dual cytoplasmic labeling of brain parenchymal cells and cellular debris. (D) Only the pan-RVFV probe labeled the ΔNSs infected mouse brain. In the inset mag, as seen in (B), only pan-RVFV signal is present in the cytoplasm of a likely microglial cell. The bar for the main images is 200 μm and for insets is 25 μm.

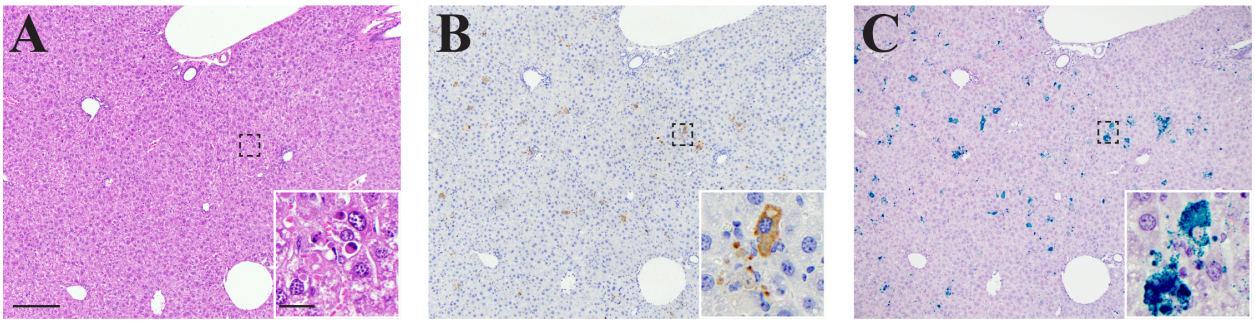

Figure 3.4 ISH and IHC labeling align with RVFV lesions in ΔNSm infected liver

Serial sections of mouse liver infected with Ken06 show that IHC and ISH labeling align with RVFV lesions. (A) H&E, inset is a magnification of dashed box region (mag), showing a typical RVFV hepatic necrosis lesion. (B) IHC using a rabbit anti-RVFV nucleocapsid polyclonal antibody (brown). In the mag, RVFV antigen labeling of a hepatocyte is diffuse and cytoplasmic. Additionally, there is multifocal labeling within the cytoplasm of an adjacent macrophage and of cellular debris. (C) The pan-RVFV probe (green) labeling is similar to but stronger than the IHC labeling. In the mag, the hepatocyte and the macrophage are strongly and nearly diffusely labeled throughout their cytoplasm and adjacent hepatocytes have scattered, punctate intracytoplasmic labeling. The bar for the main images is 200 µm and for insets is 25 µm.

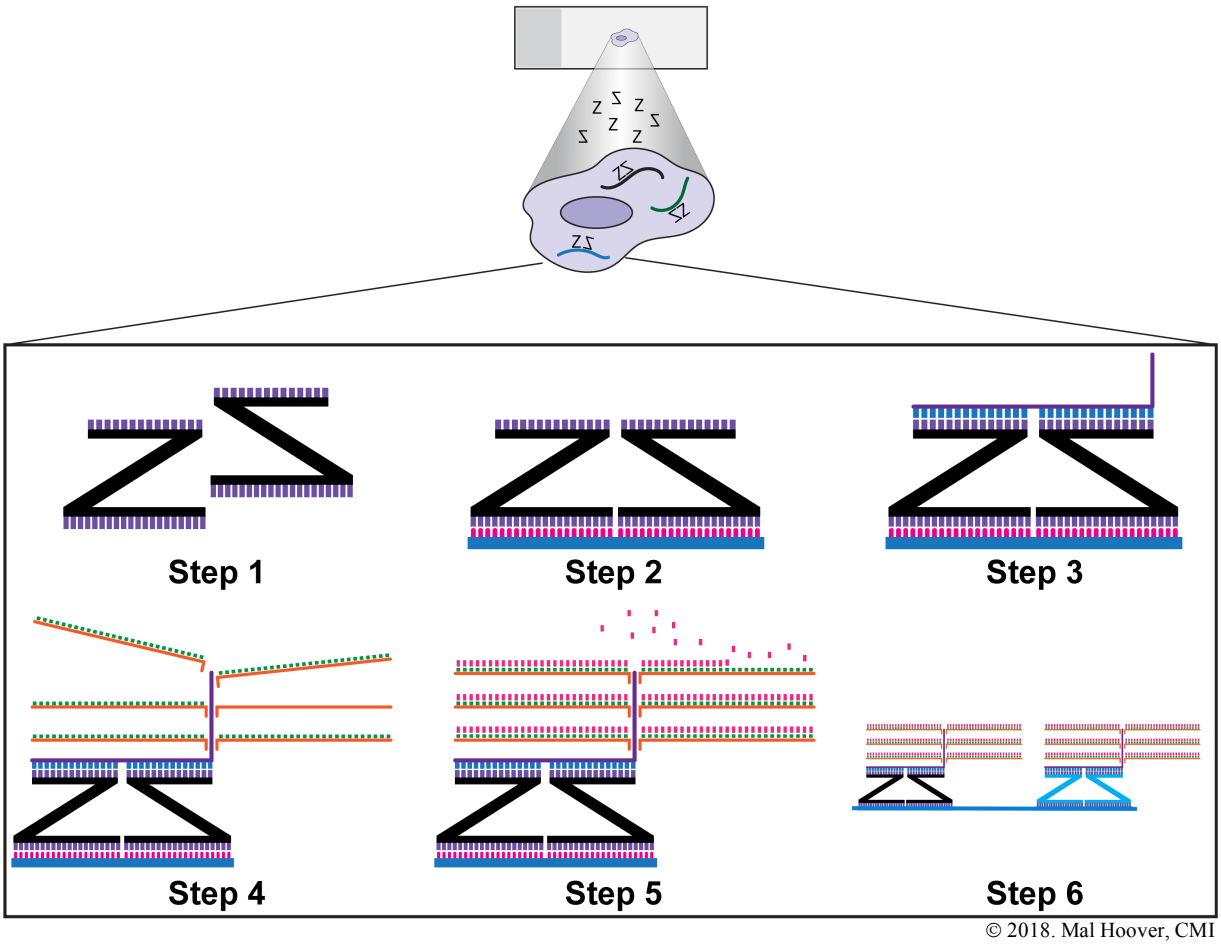

Figure 3.5 RNAscope ISH novel z probe design.

The RNAscope probes have a lower region of 18-25 bp complementary to the target RNA, a spacer sequence, and a 14 bp upper region (Step 1). To improve signal-to-noise ratio, two independent probes must hybridize to the target sequence contiguously to form a 28 bp base for signal amplification (Step 2). A pre-amplifier hybridizes to the 28 bp base (Step 3). A series of amplification steps adds amplifiers (Step 4) and labeled probes (Step 5) to the preamplifier for chromogenic detection. For each RNA target, about 20 z probe pairs (Step 6) are designed for accurate detection.

Table 3.1 Correlation between IHC, RT-PCR and ISH results on FFPE tissues

					IHC	RT-PCR (Ct)			ISH	
	Species	**Tissue**	**RVFV virus strain**	**DPI**	**Signal**	**L**	**M**	**S**	**Probe**	**Results**
Singleplex Pan-RVFV	sheep(206)	liver	Ken06	4	+	21.7	21.5	23.6	L	+
	sheep(206)	liver	Ken06	5	+	23.5	23.7	26.2	L	+
	cattle(207)	liver	Ken06	3	+	21.3	22.2	22.7	L	+
	sheep	liver	Uninfected	N/A	-	*	*	*	L	-
	sheep(206)	lymph node	Ken06	5	+	35.5	28.4	ND	L	+
	sheep(206)	kidney	Ken06	10	-	ND	33.9	ND	L	-
Duplex ΔNSm	mouse(17)	liver	ZH501-NSm-del21/384	3	+	22.3	21.6	22.6	L/M	+ / -
	mouse(277)	liver	Ken06	3	+	26.1	25.2	31.5	L/M	+ / +
	mouse(277)	liver	Mock Infected	10	-	35.39	ND	ND	L/M	- / -
Duplex ΔNSs	mouse(17)	brain	ZH501-NSs-del16/198	11	+	25.40	23.9	ND	L/S	+ / -
	mouse(277)	brain	Ken06	10	+	*	*	*	L/S	+ / +
	mouse(277)	liver	Ken06	3	+	26.06	25.2	31.5	L/S	+ / +
	mouse(277)	brain	Mock Infected	10	-	*	*	*	L/S	- / -

DPI = Days post inoculation, Ct = cycle threshold, ND = not detected, N/A = non-applicable, * = not done

Chapter 4 - Rift Valley Fever Virus Proteins Detected by Multiple Immunohistochemical Methods

Preface

This chapter is based on a collaborative effort. My contribution was optimization of western blot technique and final western blot testing of all antibodies as well as all work with the anti-NSs polyclonal antibody. Initial western blot development, immunohistochemistry, and immunofluorescence work was done by Monica Gamez and Elizabeth Stietzle. This work will be submitted for peer review and publication under the title:

Rift Valley Fever Virus Detected by Multiple Immunodetection Methods

M Gamez[1]*, I.K. Ragan[1]*, E. Stietzle[1], J.A. Richt[1], B.S. Drolet[2], W.C. Wilson[2], and A.S. Davis[1]

[1]Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS; [2]Arthropod-Borne Animal Diseases Research Unit, USDA, ARS, Manhattan, KS, USA

*These authors contributed equally to this work

Introduction

RVFV is a single-stranded, negative-sense RNA virus from the order *Bunyavirales*, family *Phenuivirdae*, genus *Phlebovirus* (278). Like other bunyaviruses, the RVFV genome is comprised of three segments; large (L), medium (M), and small (S) (4). The L segment (~6.4kb) encodes for the RNA-dependent RNA polymerase. The M segment (~3.8kb) encodes for two glycoproteins, aminoterminal glycoprotein (Gn) and carboxyterminal glycoprotein (Gc), and two minor proteins, a 78-kDa nonstructural protein and the 14-kDa nonstructural protein (NSm). The S segment (~1.7k) is an ambisense segment that encodes the nucleoprotein (N) and the nonstructural protein (NSs). The RVFV nucleocapsid and glycoproteins are considered to be strongly immunogenic (174) and have become important protein targets for vaccine and diagnostic assay development.

Although functional studies have been done on several of the viral components, much is still unknown regarding RVFV receptor binding and intracellular trafficking. Its tissue tropism, localization within tissues, and association with different cell types during infection, are not fully understood (279, 280). This limited knowledge is in part due to a lack of research tools needed to perform the necessary studies. Various in-house made monoclonal antibodies (mAbs) and polyclonal antibodies (pAbs) against different RVFV antigens have been produced and are valuable for use in enzyme-linked immunosorbent assays (ELISAs) (9, 182, 183). Nevertheless, antibodies made in-house require extensive evaluation before routine use. Readily-available antibodies raised against RVFV are accessible for research through the Biodefense and Emerging Infections Research Resources Repository (BEI Resources) but their functional activity has been limited to testing in immunofluorescence (IF) and ELISA assays (281). There is a need for working immunoassay protocols using existing antibodies against RVFV.

A panel of mAbs and pAbs raised against RVFV N, Gn, Gc, and NSs was characterized using several common immunodetection assays. The evaluation included three mAbs against N, six against Gc, and five against Gn all raised in mouse. Additionally, two pAbs against N and one against NSs raised in rabbit were evaluated. All antibodies were screened by western blot (WB) against RVFV MP12 strain-infected cell lysates. Antibodies with reactivity by WB were then further tested by immunohistochemistry (IHC) in wildtype RVFV-infected formalin-fixed, paraffin-embedded (FFPE) tissues as well as by IF in MP12-infected cells and FFPE tissues. These antibodies were used to successfully establish working protocols for each immunoassay. Throughout, the objective was to identify a set of reliable, multipurpose antibodies, at least one per target for every immunoassay and where possible to identify a universal antibody for a given antigen. This effort provides a useful set of diagnostic tools for RVFV pathogenesis studies as well as for the development of RVFV therapeutics and diagnostics.

Materials and Methods

Cells and Tissues

African green monkey kidney Vero 76 cells (ATCC; Manassas, VA) were used to produce RVFV infected and uninfected control cell lysates for the WB work. Vero MARU cells, a clone from the Middle America Research Unit, were used for all the IHC assays. All cells were cultured in growth media, Dulbecco's Modified Eagle's Media (ATCC; Manassas, VA) supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin (ThermoFisher Scientific; Waltham, MA), and maintained at 37°C, 5% CO_2. The FFPE infected and uninfected sheep liver tissues used in the IF and IHC assays came from a prior experimental challenge study with RVFV strain Kenya-128B-15 (Ken06) (206).

Viral Infections

All *in vitro* infection experiments used the RVFV vaccine strain MP12. For the immunocytochemistry (ICC) assays, cells were grown to 25% confluency on 8-well chamber slides, infected at a multiplicity of infection (MOI) of 1.0, incubated at 37°C, 5% CO_2 in growth media for 24 hours (hr), and fixed in room temperature (RT) 4% paraformaldehyde for 10 minutes (min). For WB cell lysate production, cells were grown to 95% confluency using growth media, infected with MP12 at an MOI of 1.0, incubated as above, and harvested at 24 hr post-infection using 1 mL of RIPA buffer (25mM Tris-HCl, 150mM NaCl, 1% NP40, 1% SDS, 1% Sodium Deoxycholate, pH 7.6) (ThermoFisher Scientific; Waltham, MA). Uninfected and vesicular stomatitis virus (Indiana strain L134-85)-infected cell lysates were collected as described for MP12 and used as WB controls.

Antibodies

Table 4.1 provides source information for all 17 antibodies that were evaluated. All mouse mAbs except for N1 and N2 were obtained from BEI Resources (Manassas, VA). N1 and N2 were purchased from Maine Biotechnology Services (Portland, Maine). These antibodies were originally created through a cooperative research and development agreement with the USDA ARS Arthropod Borne Animal Diseases Research Unit (ABADRU) (Manhattan, KS). Rabbit pAbs N4 and N5 were previously produced and available from ABADRU (143). PAb against NSs was kindly provided by Dr. Tetsuro Ikegami at the University of Texas Medical Branch. Unless otherwise specified, the working antibody dilutions for each assay are listed in Table 4.1.

Western blot

To separate viral proteins for WB, 20 µl of each sample was loaded on 10% Mini-PROTEAN TGX stain-free protein gel (Bio-Rad; Hercules, CA) and run at 250V for 25 min under denaturing conditions. The proteins were transferred onto 0.2 mm PVDF membrane using the TransBlot Turbo transfer system (Bio-Rad; Hercules, CA) at 1.3A for 5 min. The membranes were blocked with 5% non-fat milk in phosphate buffered saline with 0.1% Tween 20 (PBSt) for 1 hr at 4C, washed three times with PBSt, and incubated at 4°C overnight with primary antibody diluted in 1% milk in PBSt as per Table 4.1. The membranes were washed three times with PBSt, incubated for 1 hr at RT with secondary antibody diluted in 1% milk in PBSt. Peroxidase-conjugated AffiniPure donkey anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories; West Grove, PA) diluted to 1:10,000 in 1% milk in PBSt was used for the detection of all mAbs. Peroxidase-conjugated AffiniPure donkey anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories; West Grove, PA) diluted to 1:10,000 in 1% milk in PBSt was used to detect all pAbs. Membranes were washed three times with PBSt and Clarity ECL Western Blotting Substrate (Bio-Rad; Hercules, CA) applied for 5 min at RT. All the immunoblots were visualized using the ChemiDoc Touch Imaging System with Image Lab version 6.0 software (Bio-Rad; Hercules, CA). Any primary antibodies that did not detect RVFV antigen initially were further evaluated using a more sensitive avidin/biotin complex detection method, Vectastain Elite ABC-HRP kit (Vector Laboratories; Burlingame, CA). VSV-infected cells and mock (non-infected) Vero cells were used as negative controls to screen for antibody cross-reactivity. Primary mouse anti-β-Actin mAb (#3700, clone 8H10D10, Cell Signaling Technology; Danvers, MA) diluted to 1:5000 in 1% milk in PBSt served as a positive control.

Immunohistochemistry

The FFPE tissues were sectioned at 4 µm and placed on positively charged slides. Tissues were deparaffinized in xylenes and rehydrated through graded alcohols from 100%-70% to distilled water. Tissues were antigen retrieved using a vegetable steamer technique in 100°C pH 6.0 sodium citrate buffer for 20 min, then cooled to RT for 15 min, blocked with 3% hydrogen peroxide diluted in DiH20 for 10 min, and briefly rinsed with Tris Buffered Saline with 0.01% Tween-20 added (TBSt). The primary antibodies were diluted in PBSt according to Table 4.1 and incubated overnight at 4°C. An avidin-biotin complex detection system was used for all tissues, Vectastain Mouse Elite ABC kit or Vectastain Rabbit Elite ABC Kit (Vector Laboratories; Burlingame, CA) according to the manufacturer's instructions for mouse and rabbit raised antibodies respectively. The chromogen, 3,3' diaminobenzidine (DAB) was applied according to the manufacturer's instructions. The tissues were then washed with distilled water and counterstained with Mayer's hematoxylin (Electron Microscopy Sciences (EMS); Hatfield, PA), dehydrated and mounted with Permount (EMS). Images were captured and processed as described previously (206). All images were color calibrated using ChromaCal software version 2.5.1 (Datacolor Inc.; Lawrenceville, NJ).

Immunofluorescence on paraffin-embedded tissue and cells

For IF on tissues, infected and uninfected FFPE sheep liver tissues were sectioned, rehydrated, and antigen retrieved as previously described for IHC. IF on cells was performed on RVFV infected and uninfected Vero cells prepared as described above. The cells were permeabilized for 5 min with 0.1% Triton-X 100 in TBS and washed in TBSt. Tissues and cells were blocked for 20 min in 5% Donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in TBS, and incubated overnight at 4°C with primary antibody diluted

according to Table 4.1. The tissues and cells were washed with PBSt and secondary antibody was applied for 30 min as follows: donkey anti-mouse 594 (Jackson ImmunoResearch Laboratories; West Grove, PA) diluted to 1:200 in TBS for mAbs or donkey anti-rabbit 594 (Jackson ImmunoResearch Laboratories; West Grove, PA) diluted to 1:200 in TBS for pAbs. For Gc1, donkey anti-mouse biotin-conjugated secondary (Jackson ImmunoResearch Laboratories; West Grove, PA) was diluted to 1:200 and applied for 30 min followed by streptavidin Alexa Fluor 568 conjugate (Thermo Fisher Scientific; Grand Island, NY) for 30 min. Nuclei were stained with DAPI (Life Technologies; Waltham, MA) and slides aqueous mounted with ProLong Gold antifade reagent (Life Technologies; Waltham, MA). Immunofluorescence images were captured on an Eclipse 90i with the DAPI and TRITC filter sets (Nikon; Melville, NJ), a Plan Apo 20x/0.75 NA objective and a Plan Apo 40x/0.95 NA objective, for tissues and cells, respectively. All images for a given target, tissue or cells, were captured using equivalent microscope settings unless otherwise noted. Additionally, adjustments made in Imaris version 9.0 (Bitplane; Zurich, Switzerland) and Photoshop CC 2018 (Adobe; San Jose, CA) were consistent within a given protocol.

Results

Screening of mAbs and pAbs for use in WB

First, WB was used to screen all antibodies for specificity. Antibodies that reacted by WB were further serially screened by IHC and IF in line with the objective to identify multipurpose antibodies. Only when later assays failed with an initially selected antibody for a given target protein was another antibody selected and tested. We identified that the two pAbs against RVFV N (N4, N5), the pAb against NSs, four mAbs against Gn (Gn1, Gn2, Gn4, Gn5), two mAbs

against Gc (Gc1, Gc2), and two mAbs against N (N1, N2) worked in our WB protocols. Fig. 4.1 shows reactivity of N4, Gn2, Gc2 and NSs against infected cell lysates. Because N5 had a weak signal, an avidin-biotin complex detection system was used to further amplify N5 for the detection of RVFV N. Optimized dilutions for each antibody are listed in Table 4.1. All antibodies that did react to MP12 cell lysate gave a single, specific band at the appropriate molecular size for their respective target.

Screening of mAbs and pAbs for use in IHC on FFPE Tissues

A subset of mAbs and pAbs (N2, N4, N5, Gn2, Gc2, NSs) that reacted on WB were screened by IHC for their efficacy on FFPE tissues. Efficacy of the antibodies for IHC is summarized in Table 4.1. These antibodies yielded minimal to no background on uninfected tissues. Fig. 4.2 shows IHC results for the detection of all four RVFV antigens using mAbs N2, Gn2, Gc2, and pAb NSs. Additionally, pAbs N4 and N5 showed similar, distinct labeling of lesions in infected tissues (data not shown).

Screening of mAbs and pAbs for use in IF on FFPE Tissues and Fixed Cells

Similar to IHC, mAbs and pAbs against all four antigens were screened for efficacy by IF on FFPE sheep tissue and fixed cells. Efficacy of the antibodies for IF is summarized in Table 4.1. Fig. 4.3 shows strong labeling of all four RVFV antigen targets in infected FFPE tissues with N5, Gn2, Gc2, and NSs. N2 also labeled RVFV in tissues (data not shown). In Fig. 4.4, antibodies Gc2, Gn1, N2, and NSs showed strong labeling in the cytoplasm of MP12 infected cells. Gc1 and N1 also produced similar results (data not shown). The two rabbit pAb (N4, N5) aberrantly labeled intra-nuclearly.

Discussion

Reliable antibodies raised against RVFV antigens are useful for both RVFV research and diagnostic applications. We evaluated multiple mAbs and pAbs raised against four different RVFV antigens (N, NSs, Gn, and Gc) for their ability to specifically recognize the presence or absence of their target viral antigen in a variety of immunodetection assays. Methods were developed to use these antibodies in WB, FFPE IHC, and IF on FFPE tissues and fixed cells. These methods have applications in research, diagnostic development, and pathogenesis studies.

Several readily-available pAbs or mAbs have been used to detect the presence and distribution of RVFV viral antigen in infected tissues. For example, mAb Gn2 (clone 4D4) has been extensively used for RVFV detection. For IHC, Gn2 labels antigen in liver, spleen, tonsil, and lymph node from lambs (282) as well as mouse brain (202). Additionally, Gn2 was successfully used in ICC (159, 174, 283, 284). Gn2 was also used in WB to demonstrate antigenicity of recombinant RVFV proteins (174). Another commonly used antibody is mAb N3 (clone 1D8), which labeled antigen in WB (174, 285) and in ICC (159, 283, 284). Finally, the successful use of a similarly produced but different pAb against the N protein was previously described in ICC and in IHC with infected sheep liver as well as cattle liver and brain tissues (143).

The detection of RVFV with various immunohistochemical methods using mAb N3, mAb Gn2, and pAb N demonstrates the consistency and reliability of these antibodies. Our evaluation identified additional anti-N and anti-Gn antibodies that successfully detected RVFV. Furthermore, we tested mAbs and pAbs against other RVFV antigens that have not yet been evaluated in WB, IF, and IHC. Specifically, anti-Gc and anti-NSs antibodies are untested for

IHC. We expanded upon the tools available for RVFV detection by providing working protocols using antibodies against N, Gn, Gc, and NSs.

All antibodies were initially screened by WB. If no signal was detected by WB, the antibody was set aside and not revisited unless no other working options were later found for other assays. Consequently, not all the antibodies were exhaustively tested in all immunodetection assay types. None of the antibodies showed cross-reactivity with VSV-infected or mock-infected cell lysates by WB. Additional testing should be done using non-RVFV *Phleboviruses* to ensure the antibodies' specificity for RVFV.

After WB screening, a subset of antibodies (N2, Gn2, Gc2, NSs) to each of the four RVFV antigens was further screened by IHC. PAbs N4 and N5 were also included for a comparison between mAb and pAb detection using IHC. As expected the pAbs, especially N4, were stronger in signal than the mAbs. For all these antibodies, the labeling of RVFV antigen in infected tissue is consistent with previous findings (143, 206).

A subset of antibodies (N1, N2, N4, N5, Gn1, Gn2, Gc1, Gc2, NSs) to each of the four RVFV antigens was also tested by IF in tissues and cells. The labeling pattern seen in the cells and tissues was consistent with previously published studies in which viral antigens were identified in the cytoplasmic compartment of infected cells (160, 174, 283, 286, 287). N2, N5, Gn2, Gc2, and NSs were identified to be successful in WB, IHC and IF.

It is important to note that the IHC and IF FFPE tissue methods reported here are for sheep liver samples only. Other species and tissue targets require additional validation. These methods have been adapted for a subset of these antibodies for use in different ruminant tissues including brain, lymph node, kidney, and lung (177, 206, 207). At times this has required the use of different chromogen and counterstain combinations to differentiate the RVFV antigen signal

from natural and disease-linked pigments, such as melanin and hemosiderin respectively. VIP chromogen (Vector Laboratories), which is violet in combination with methyl green counterstain, enabled differentiation of viral antigen from hemosiderin deposits in lymph nodes (177).

Access to widely available, validated antibodies raised against various RVFV proteins will help standardize techniques for RVFV detection. Ideally, these antibodies should be commercialized and produced in bulk to minimize inter-lot variations (288). While N2 initially worked well in WB, continued use of multiple lots revealed inconsistencies in this antibody's reliability for use in immunodetection assays. Attempts to improve N2's stability and reliability with cryoprotectants and preservatives failed. This reinforces the importance of screening replacement antibody lots against prior ones in order to ensure continuity of assays.

Conclusion

RVFV is a significant mosquito-borne pathogen that can spread from animals to humans causing severe disease and mortality. Currently, it is classified as an overlap Select Agent and category A priority pathogen by the USDA and CDC. Consequently, ongoing research is focused on understanding RVFV pathogenesis in order to develop preventative measures like effective vaccines and accurate diagnostic tests. The antibodies and associated immunodetection assay methods presented here provide a useful set of tools to support these research and diagnostic needs.

Acknowledgements

The following reagents were obtained from Joel M. Dalrymple-Clarence J. Peters USAMRIID Antibody Collection through BEI Resources, NIAID, NIH: Monoclonal Anti-Rift Valley Fever Virus Gn Glycoproteins (Clone 4D4, NR-43190) (Clone 439CC, NR-43195) (Clone 3C10, NR-43186) (Clone 7B6, NR-43185) (Clone 3D11, NR-43189), Gc Glycoproteins (Clone 4B6, NR-43184) (Clone 5D2, NR-43191) (Clone 5E2, NR-43192) (Clone 1F6, NR-43193) (Clone 1G4, NR-43738) (Clone 9C10, NR-43187), and N Nucleocapsid (Clone 1D8, NR-43188). Monoclonal anti-RVFV N (N1 and N2) were kindly provided by Dr. Scott McVey (USDA ARS Arthropod-Borne Animal Disease Research Unit, Manhattan, KS). Polyclonal anti-RVFV NSs was kindly provided by Dr. Tetsuro Ikegami (University of Texas Medical Branch, Galveston, TX). The vesicular stomatitis virus was provided by Robert Tesh (University of Texas Medical Branch, Galveston, TX). The authors thank the Kansas State University Veterinary Diagnostic Laboratory Histopathology Unit for their assistance with tissue sectioning. This work was funded by the US Department of Agriculture, Agricultural Research Service (3020-32000-009-00D) and the Department of Diagnostic Medicine, College of Veterinary Medicine, Kansas State University's startup funds for Dr. Davis. The authors declare no competing interests.

Table 4.1 RVFV antibody evaluation by WB, IHC, and IF

Antibody name	Name	Clone #	Clonality	Animal Host	Source	WB	Enhancing step for WB	IHC	IF in tissue	IF in cells
N1	MAB239P	06C10	monoclonal	Mouse	MBS	1:1000	*	*	*	1:500
N2	MAB240P	09F04	monoclonal	Mouse	MBS	1:1000	*	1:1000	1:500	1:500
N3	NR43188	1D8	monoclonal	Mouse	BEI (NIH)	ND	*	*	*	*
N4	Rabbit Ab #2		polyclonal	Rabbit	ABADRU	1:5000	*	1:500	*	ND (nuclear)
N5	Rabbit Ab #3		polyclonal	Rabbit	ABADRU	1:500	yes	1:500	1:200	ND (nuclear)
Gn1	NR43195	4-39-CC	monoclonal	Mouse	BEI (NIH)	1:1000	*	*	*	1:200
Gn2	NR43190	4D4	monoclonal	Mouse	BEI (NIH)	1:500	*	1:1000	1:500	ND
Gn3	NR43186	3C10	monoclonal	Mouse	BEI (NIH)	ND	no	*	*	ND
Gn4	NR43185	7B6	monoclonal	Mouse	BEI (NIH)	1:500	*	*	*	*
Gn5	NR43189	3D11	monoclonal	Mouse	BEI (NIH)	1:500	*	*	*	*
Gc1	NR43184	4B6	monoclonal	Mouse	BEI (NIH)	1:1000	*	*	*	1:200
Gc2	NR43191	5D2	monoclonal	Mouse	BEI (NIH)	1:500	*	1:500	1:200	1:200
Gc3	NR43192	5E2	monoclonal	Mouse	BEI (NIH)	ND	no	*	*	*
Gc4	NR43193	1F6	monoclonal	Mouse	BEI (NIH)	ND	no	*	*	*
Gc5	NR43738	1G4	monoclonal	Mouse	BEI (NIH)	ND	no	*	*	*
Gc6	NR43187	9C10	monoclonal	Mouse	BEI (NIH)	ND	no	*	*	*
NSs	NSs		polyclonal	Rabbit	UTMB	1:2000	*	1:500	1:500	1:1000

MBS= Maine Biotechnology Services

ND= no signal detected

*= not done

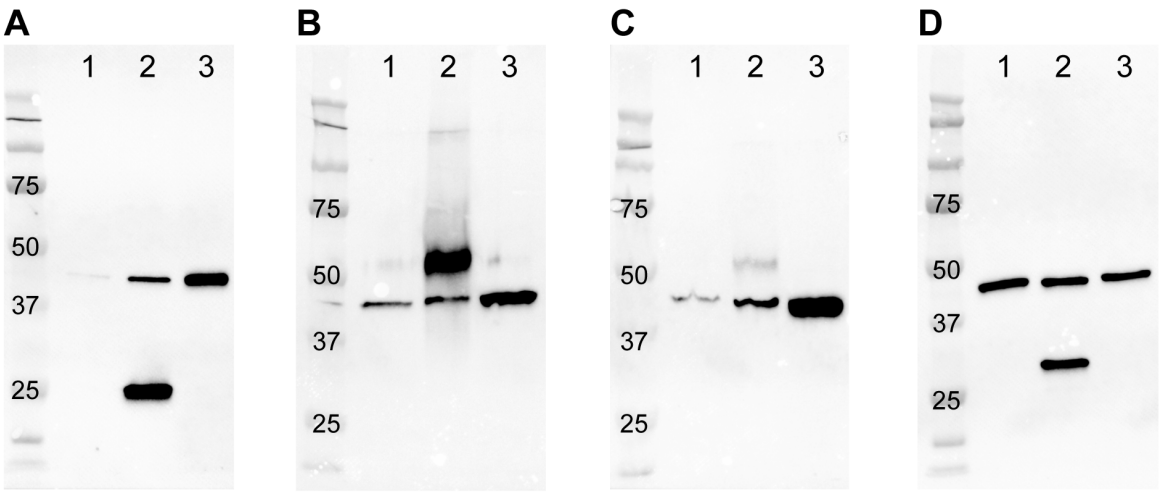

Figure 4.1 WB analysis of mAb and pAb specificity against MP12 infected cell lysate.

Each antibody was tested with the following samples; lane 1= mock-infected Vero cells, lane 2 = MP12 infected cell lysate, lane 3 = VSV infected cell lysate. The molecular size markers in kDa are on the left. A single band in the target appropriate location was consistently detected in the MP12 cell lysate using (A) N4 (29 kDa), (B) Gn2 (54 kDa), (C) Gc2 (60kDa), and (D) NSs (34kDa). The antibodies showed no reactivity to mock (non-infected) or VSV-infected cell lysate. The anti-β-actin mAb used as a protein loading control showed distinct and expected bands at 42 kDa for all wells.

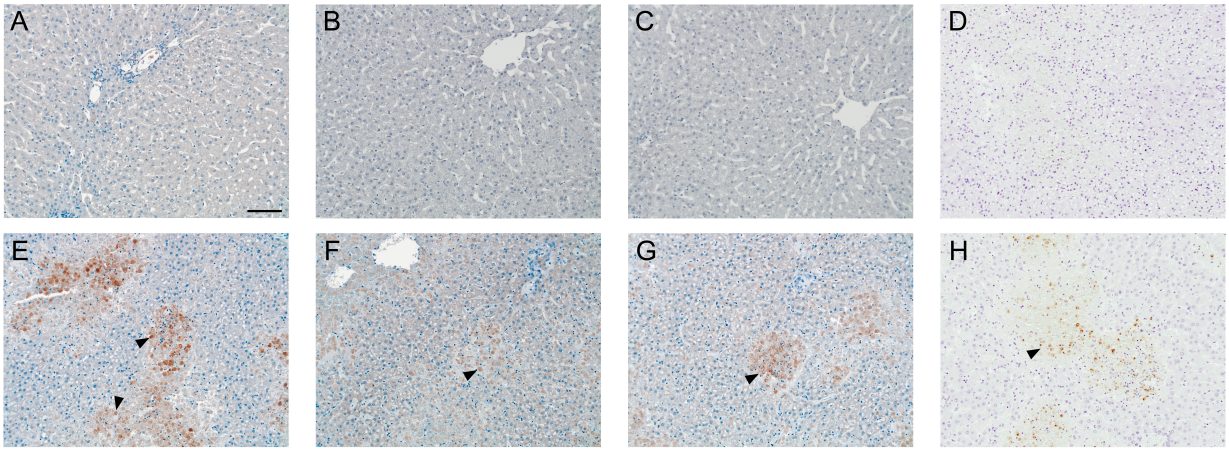

Figure 4.2 RVFV antigen IHC on infected FFPE liver tissue.

Uninfected and RVFV Ken06 infected FFPE sheep liver sections were labeled with individual anti-RVFV protein mAbs using an avidin-biotin complex technique with DAB chromogen and a hematoxylin counterstain. Row 1: None of the RVFV antibodies labeled uninfected sheep liver: (A) mAb anti-RVFV N2, (B) mAb anti-RVFV Gc2 (C) mAb anti-RVFV Gn2, (D) pAb anti-RVFV NSs. Row 2: At least one antibody raised against each target RVFV protein labeled infected sheep liver: (E) mAb anti-RVFV N2, (F) mAb anti-RVFV Gc2 (G) mAb anti-RVFV Gn2, (H) pAb anti-RVFV NSs. The arrowheads denote infected cells, some which still retain their nucleus, and positive cellular debris. The cells were primarily hepatocytes and lesser numbers of macrophages. Bar is 100 μm.

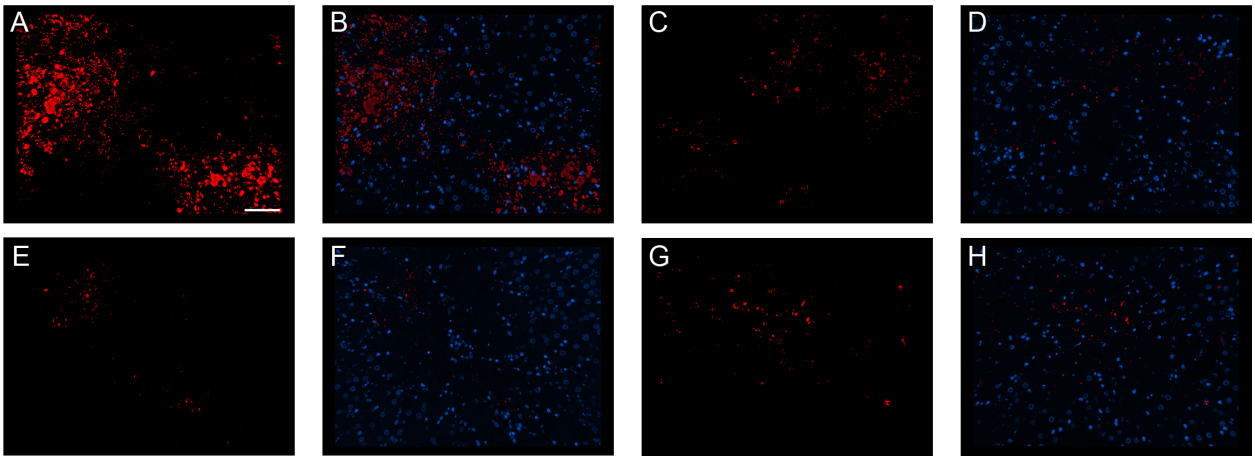

Figure 4.3 RVFV antigen IF on infected FFPE sheep liver tissue.

Uninfected and Ken06 infected sheep liver tissue were labeled with individual anti-RVFV mAbs and visualized with either anti-mouse or rabbit Alexa Fluor 594 conjugated secondary antibody or anti-mouse biotin conjugated secondary antibody and streptavidin conjugated Alexa Fluor 568. Rows 1 and 2: Anti-RVFV labeling results with infected liver tissue: (A) pAb N5 (red), (B) merge with DAPI (blue). (C) mAb Gn2 (red), (D) merge. (E) mAb Gc2 (red), (F) merge. (G) pAb NSs, (H) merge. RVFV antigen labeling is concentrated in lesions, identifiable by the punctate nuclear debris. Bar is 30 μm.

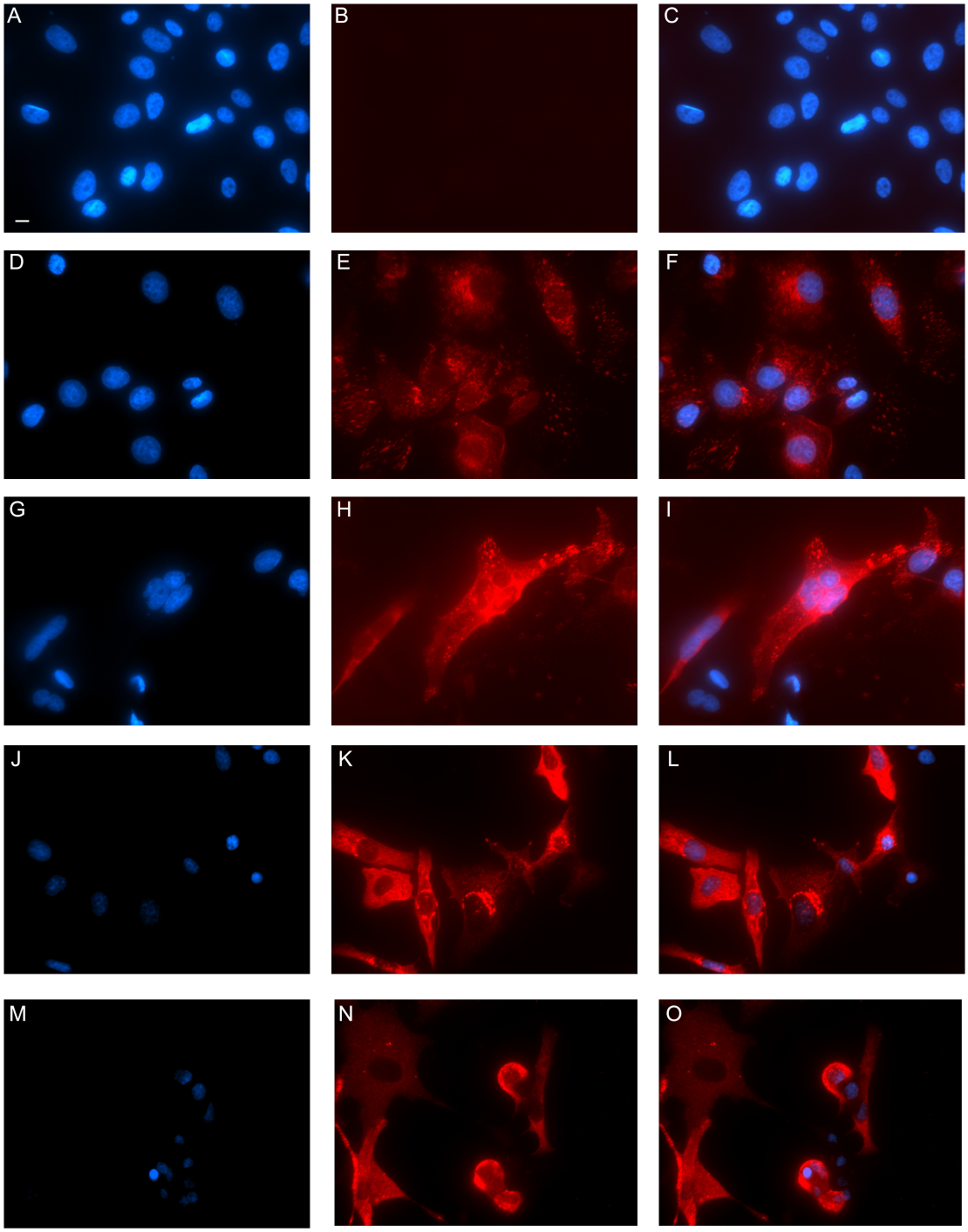

Figure 4.4 RVFV antigen IF on paraformaldehyde-fixed infected cells.

Uninfected and RVFV MP12 infected Vero cells were labeled with an individual anti-RVFV mAbs and visualized with anti-mouse Alexa Fluor 594 conjugated secondary antibody. Row 1:

Uninfected Vero cells, (A) DAPI (blue), (B) anti-RVFV Gc2 (red), (C) merge are representative of labeling of uninfected tissues by all antibodies. (D-F): Anti-RVFV Gc2 labeling of infected cells shows a cytoplasmic RVFV signal as expected, image order as prior. (G-I): Mouse mAb anti-RVFV Gn1. (J-L): Mouse mAb anti-RVFV N2. (M-O): Rabbit pAb anti-RVFV NSs. Bar is 10 μm.

Chapter 5 - Concluding Remarks

The threat of transboundary animal diseases like RVFV continues to increase as world travel and global trade grows. The battle against this re-emerging disease requires continuous development of countermeasures like vaccines, therapeutics, and especially diagnostics. Versatile and accurate diagnostic assays that can differentiate infected from vaccinated animals (DIVA) are needed to supplement vaccine programs. The research presented in this dissertation offers valuable diagnostic tools for pathogenesis and vaccine efficacy studies as well as aids in the surveillance of RVFV globally.

We developed several diagnostic tools for detecting RVFV nucleic acid, antibodies, and antigens. In Chapter 2 we evaluated a bead-based multiplexing immunoassay for the detection of IgM and IgG antibodies against RVFV Gn and N protein. Moreover, this immunoassay is DIVA compatible with a candidate RVFV Gn/Gc subunit vaccine (106, 177). In Chapter 3 we developed the first ISH assay to detect RVFV RNA in formalin-fixed, paraffin-embedded tissues. Additionally, we demonstrated the DIVA compatibility of this ISH assay to detect candidate RVFV NSm and NSs deletion vaccines (17). And in Chapter 4 we assess multiple anti-RVFV antibodies for their efficacy in a variety of assays including western blot, immunohistochemistry, and immunofluorescence. The work in this dissertation offers versatile and sensitive diagnostic tools that have applications in both endemic and non-endemic countries.

The production of innovative and readily available technologies is important for medical advancement (289). Therefore, we will continue the validation of the FMIA and ISH assays for commercial use in diagnostic laboratories. The FMIA and ISH assays described in this dissertation are in the early stages of validation. Currently, under the guidelines for diagnostic

assay development (290), the FMIA is at Stage 3 of 4 and the ISH is at Stage 2 of 4 for validation. This validation work continues.

Once validated the FMIA and ISH assays can be used for routine testing of RVFV samples in diagnostic laboratories. First, the validated FMIA RVFV antigen targets can be incorporated into a larger FMIA panel to simultaneously screen endemic and exotic ruminant diseases. For example, during an outbreak in RVFV-endemic areas, the multiplex panel could be used to screen for RVF, bluetongue, rinderpest, peste des petits ruminants, Wesselsbron viruses, and other abortive pathogens. In non-endemic areas, the panel could be used to screen samples during a foreign animal disease investigation or at ports of entry for livestock. The FMIA can replace traditional serological testing by enzyme-linked immunosorbent assay (ELISA). Ultimately, the FMIA is a versatile tool for rapid screening of multiple pathogens.

Second, the RVFV ISH assay should be a valuable tool for pathogenesis and vaccine research. Specifically, ISH can serve as a confirmatory test for RVFV molecular detection as well as for the detection of DIVA markers in candidate gene-deletion vaccines. As the RNAscope technology becomes more affordable, the assay can be used in endemic countries to test tissues such as aborted fetuses during an outbreak. Both the Luminex FMIA and RNAscope ISH platforms are designed to be user friendly and support high-throughput testing, which is important for diagnostic laboratories. In summary, the FMIA and ISH have many applications and are valuable tools in both endemic and non-endemic countries.

Despite the development of novel RVFV detection methods, there is still need for affordable RVFV diagnostic assays for routine use in endemic countries. The initial costs of the diagnostic tools described in this dissertation may be cost-prohibitive in Africa. The cost to run a full 96-well plate using the FMIA is about $20 per plate while the ISH is about $60 per slide (not

including equipment cost). Nonetheless, other RVFV molecular tests such as real time, reverse transcription PCR also require specialized equipment and the commercial ELISA kits like the ID-Vet ELISA can also be costly for resource-constrained countries. In-house assays for RVFV detection are commonly used to reduce reagent and equipment costs but they lack the proper assay validation and reliability for high-volume testing. Consequently, future diagnostic development needs to focus on high-throughput yet affordable testing methods in endemic areas.

To combat the emergence and re-emergence of infectious diseases, a more holistic or "One Health" approach is needed. The One Health Initiative expands collaborations with scientific-health and environmental fields to tackle human, animal, and environmental concerns (291). A critical factor in controlling infectious diseases involves successful national and global efforts to create effective surveillance and response systems (292). Not only does the work in this dissertation provide solutions to the immediate needs for RVFV diagnostics but it also supports the global One Health Initiative by providing new DIVA-compatible diagnostics for controlling RVFV and protecting animal and human health. Finally, the successful development of diagnostic tools against RVFV can be translated to assay development for other transboundary animal diseases.

References

1. **Mandell RB, Flick R.** 2010. Rift Valley fever virus: an unrecognized emerging threat? Hum Vaccin **6:**597-601.
2. **OIE.** 2014. OIE Terrestrial Manual. Rift Valley Fever (Infection with Rift Valley fever virus) Chapter 2114. **1**:1-21.
3. **Khan AS, Smith CV.** 2016. Rift Valley fever: still an emerging infection after 3500 years. Lancet Glob Health **4:**e773-e774.
4. **Giorgi C, Accardi L, Nicoletti L, Gro MC, Takehara K, Hilditch C, Morikawa S, Bishop DH.** 1991. Sequences and coding strategies of the S RNAs of Toscana and Rift Valley fever viruses compared to those of Punta Toro, Sicilian Sandfly fever, and Uukuniemi viruses. Virology **180:**738-753.
5. **Sherman MB, Freiberg AN, Holbrook MR, Watowich SJ.** 2009. Single-particle cryo-electron microscopy of Rift Valley fever virus. Virology **387:**11-15.
6. **Elliott RM, Schmaljohn CS, Collett MS.** 1991. Bunyaviridae genome structure and gene expression. Curr Top Microbiol Immunol **169:**91-141.
7. **Le May N, Gauliard N, Billecocq A, Bouloy M.** 2005. The N terminus of Rift Valley fever virus nucleoprotein is essential for dimerization. J Virol **79:**11974-11980.
8. **Liu L, Celma CC, Roy P.** 2008. Rift Valley fever virus structural proteins: expression, characterization and assembly of recombinant proteins. Virol J **5:**82.
9. **Fafetine JM, Tijhaar E, Paweska JT, Neves LC, Hendriks J, Swanepoel R, Coetzer JA, Egberink HF, Rutten VP.** 2007. Cloning and expression of Rift Valley fever virus nucleocapsid (N) protein and evaluation of a N-protein based indirect ELISA for the

detection of specific IgG and IgM antibodies in domestic ruminants. Vet Microbiol **121:**29-38.

10. **Le May N, Dubaele S, Proietti De Santis L, Billecocq A, Bouloy M, Egly JM.** 2004. TFIIH transcription factor, a target for the Rift Valley hemorrhagic fever virus. Cell **116:**541-550.

11. **Struthers JK, Swanepoel R.** 1982. Identification of a major non-structural protein in the nuclei of Rift Valley fever virus-infected cells. J Gen Virol **60:**381-384.

12. **Le May N, Mansuroglu Z, Leger P, Josse T, Blot G, Billecocq A, Flick R, Jacob Y, Bonnefoy E, Bouloy M.** 2008. A SAP30 complex inhibits IFN-beta expression in Rift Valley fever virus infected cells. PLoS Pathog **4:**e13.

13. **Austin D, Baer A, Lundberg L, Shafagati N, Schoonmaker A, Narayanan A, Popova T, Panthier JJ, Kashanchi F, Bailey C, Kehn-Hall K.** 2012. p53 Activation following Rift Valley fever virus infection contributes to cell death and viral production. PLoS One **7:**e36327.

14. **Lihoradova O, Ikegami T.** 2014. Countermeasure development for Rift Valley fever: deletion, modification or targeting of major virulence factor NSs. Future Virol **9:**27-39.

15. **Mudhasani R, Tran JP, Retterer C, Kota KP, Whitehouse CA, Bavari S.** 2016. Protein Kinase R Degradation Is Essential for Rift Valley Fever Virus Infection and Is Regulated by SKP1-CUL1-F-box (SCF)FBXW11-NSs E3 Ligase. PLoS Pathog **12:**e1005437.

16. **Bird BH, Albariño CG, Hartman AL, Erickson BR, Ksiazek TG, Nichol ST.** 2008. Rift valley fever virus lacking the NSs and NSm genes is highly attenuated, confers

protective immunity from virulent virus challenge, and allows for differential identification of infected and vaccinated animals. J Virol **82:**2681-2691.

17. **Nishiyama S, Slack OA, Lokugamage N, Hill TE, Juelich TL, Zhang L, Smith JK, Perez D, Gong B, Freiberg AN, Ikegami T.** 2016. Attenuation of pathogenic Rift Valley fever virus strain through the chimeric S-segment encoding sandfly fever phlebovirus NSs or a dominant-negative PKR. Virulence **7:**871-881.

18. **Kakach LT, Wasmoen TL, Collett MS.** 1988. Rift Valley fever virus M segment: use of recombinant vaccinia viruses to study Phlebovirus gene expression. J Virol **62:**826-833.

19. **Won S, Ikegami T, Peters CJ, Makino S.** 2007. NSm protein of Rift Valley fever virus suppresses virus-induced apoptosis. J Virol **81:**13335-13345.

20. **Kreher F, Tamietti C, Gommet C, Guillemot L, Ermonval M, Failloux AB, Panthier JJ, Bouloy M, Flamand M.** 2014. The Rift Valley fever accessory proteins NSm and P78/NSm-GN are distinct determinants of virus propagation in vertebrate and invertebrate hosts. Emerg Microbes Infect **3:**e71.

21. **Bird BH, Albarino CG, Nichol ST.** 2007. Rift Valley fever virus lacking NSm proteins retains high virulence in vivo and may provide a model of human delayed onset neurologic disease. Virology **362:**10-15.

22. **Weingartl HM, Zhang S, Marszal P, McGreevy A, Burton L, Wilson WC.** 2014. Rift Valley fever virus incorporates the 78 kDa glycoprotein into virions matured in mosquito C6/36 cells. PLoS One **9:**e87385.

23. **Rusu M, Bonneau R, Holbrook MR, Watowich SJ, Birmanns S, Wriggers W, Freiberg AN.** 2012. An assembly model of rift valley Fever virus. Front Microbiol **3:**254.

24. **Besselaar TG, Blackburn NK.** 1991. Topological mapping of antigenic sites on the Rift Valley fever virus envelope glycoproteins using monoclonal antibodies. Arch Virol **121:**111-124.

25. **Zamoto-Niikura A, Terasaki K, Ikegami T, Peters CJ, Makino S.** 2009. Rift valley fever virus L protein forms a biologically active oligomer. J Virol **83:**12779-12789.

26. **Reguera J, Weber F, Cusack S.** 2010. Bunyaviridae RNA polymerases (L-protein) have an N-terminal, influenza-like endonuclease domain, essential for viral cap-dependent transcription. PLoS Pathog **6:**e1001101.

27. **Anyangu AS, Gould LH, Sharif SK, Nguku PM, Omolo JO, Mutonga D, Rao CY, Lederman ER, Schnabel D, Paweska JT, Katz M, Hightower A, Njenga MK, Feikin DR, Breiman RF.** 2010. Risk factors for severe Rift Valley fever infection in Kenya, 2007. Am J Trop Med Hyg **83:**14-21.

28. **Adam I, Karsany MS.** 2008. Case report: Rift Valley Fever with vertical transmission in a pregnant Sudanese woman. J Med Virol **80:**929.

29. **Nicholas DE, Jacobsen KH, Waters NM.** 2014. Risk factors associated with human Rift Valley fever infection: systematic review and meta-analysis. Trop Med Int Health **19:**1420-1429.

30. **Hartman AL, Powell DS, Bethel LM, Caroline AL, Schmid RJ, Oury T, Reed DS.** 2014. Aerosolized rift valley fever virus causes fatal encephalitis in african green monkeys and common marmosets. J Virol **88:**2235-2245.

31. **NIAID.** NIAID Emerging Infectious Diseases/Pathogen. https://www.niaid.nih.gov/research/emerging-infectious-diseases-pathogens. Accessed

32. **Rolin AI, Berrang-Ford L, Kulkarni MA.** 2013. The risk of Rift Valley fever virus introduction and establishment in the United States and European Union. Emerg Microbes Infect **2:**e81.

33. **Daubney R, Hudson JR.** 1931. Enzootic Hepatitis or Rift Valley Fever. An undescribed virus disease in sheep cattle and man from East Africa. The Journal of Pathology and Bacteriology **34:**545-579.

34. **Gerdes GH.** 2004. Rift Valley fever. Rev Sci Tech **23:**613-623.

35. **CDC.** 2017. Rift Valley Fever (RVF) Outbreak Summaries, *on* CDC. https://www.cdc.gov/vhf/rvf/outbreaks/summaries.html. Accessed 09/10/2017.

36. **Nguku PM, Sharif SK, Mutonga D, Amwayi S, Omolo J, Mohammed O, Farnon EC, Gould LH, Lederman E, Rao C, Sang R, Schnabel D, Feikin DR, Hightower A, Njenga MK, Breiman RF.** 2010. An investigation of a major outbreak of Rift Valley fever in Kenya: 2006-2007. Am J Trop Med Hyg **83:**5-13.

37. **Shoemaker T, Boulianne C, Vincent MJ, Pezzanite L, Al-Qahtani MM, Al-Mazrou Y, Khan AS, Rollin PE, Swanepoel R, Ksiazek TG, Nichol ST.** 2002. Genetic analysis of viruses associated with emergence of Rift Valley fever in Saudi Arabia and Yemen, 2000-01. Emerg Infect Dis **8:**1415-1420.

38. **Chevalier V, Pepin M, Plee L, Lancelot R.** 2010. Rift Valley fever--a threat for Europe? Euro Surveill **15:**19506.

39. **Abd el-Rahim IH, Abd el-Hakim U, Hussein M.** 1999. An epizootic of Rift Valley fever in Egypt in 1997. Rev Sci Tech **18:**741-748.

40. **Zeller HG, Fontenille D, Traore-Lamizana M, Thiongane Y, Digoutte JP.** 1997. Enzootic activity of Rift Valley fever virus in Senegal. Am J Trop Med Hyg **56:**265-272.

41. **de La Rocque S, Formenty P.** Rift Valley Fever, Disease Ecology and Early Warning, *In* Odongo N, Garcia M, Viljoen G (ed): 327-333.

42. **Balenghien T, Cardinale E, Chevalier V, Elissa N, Failloux AB, Jean Jose Nipomichene TN, Nicolas G, Rakotoharinome VM, Roger M, Zumbo B.** 2013. Towards a better understanding of Rift Valley fever epidemiology in the south-west of the Indian Ocean. Vet Res **44:**78.

43. **CDC.** 08/8/2016 2016. Cost of the Ebola Epidemic. https://www.cdc.gov/vhf/ebola/outbreaks/2014-west-africa/cost-of-ebola.html. Accessed 01/24/2018.

44. **Rich KM, Wanyoike F.** 2010. An assessment of the regional and national socio-economic impacts of the 2007 Rift Valley fever outbreak in Kenya. Am J Trop Med Hyg **83:**52-57.

45. **Shimshony A, Economides P.** 2006. Disease prevention and preparedness for animal health emergencies in the Middle East. Rev Sci Tech **25:**253-269.

46. **Hartley DM, Rinderknecht JL, Nipp TL, Clarke NP, Snowder GD, Fever NCfFAaZDDAGoRV.** 2011. Potential effects of Rift Valley fever in the United States. Emerg Infect Dis **17:**e1.

47. **Shiferaw ML, Doty JB, Maghlakelidze G, Morgan J, Khmaladze E, Parkadze O, Donduashvili M, Wemakoy EO, Muyembe JJ, Mulumba L, Malekani J, Kabamba J, Kanter T, Boulanger LL, Haile A, Bekele A, Bekele M, Tafese K, McCollum AA,**

Reynolds MG. 2017. Frameworks for Preventing, Detecting, and Controlling Zoonotic Diseases. Emerg Infect Dis **23**.

48. **Borio L, Inglesby T, Peters CJ, Schmaljohn AL, Hughes JM, Jahrling PB, Ksiazek T, Johnson KM, Meyerhoff A, O'Toole T, Ascher MS, Bartlett J, Breman JG, Eitzen EM, Jr., Hamburg M, Hauer J, Henderson DA, Johnson RT, Kwik G, Layton M, Lillibridge S, Nabel GJ, Osterholm MT, Perl TM, Russell P, Tonat K, Working Group on Civilian B.** 2002. Hemorrhagic fever viruses as biological weapons: medical and public health management. JAMA **287:**2391-2405.

49. **Miller W, Demach P, Rosenberger C, Dominik J, Bradshaw J.** 1963. Stability and Infectivity of Airborne Yellow Fever and Rift Valley Fever Viruses. The American Journal of Hygiene **77:**114-121.

50. **Baba M, Masiga DK, Sang R, Villinger J.** 2016. Has Rift Valley fever virus evolved with increasing severity in human populations in East Africa? Emerg Microbes Infect **5:**e58.

51. **Anyamba A, Chretien JP, Small J, Tucker CJ, Formenty PB, Richardson JH, Britch SC, Schnabel DC, Erickson RL, Linthicum KJ.** 2009. Prediction of a Rift Valley fever outbreak. Proc Natl Acad Sci U S A **106:**955-959.

52. **Ikegami T, Makino S.** 2011. The pathogenesis of Rift Valley fever. Viruses **3:**493-519.

53. **Ross TM, Bhardwaj N, Bissel SJ, Hartman AL, Smith DR.** 2012. Animal models of Rift Valley fever virus infection. Virus Res **163:**417-423.

54. **Keefer GV, Zebarth GL, Allen WP.** 1972. Susceptibility of dogs and cats to Rift Valley fever by inhalation or ingestion of virus. J Infect Dis **125:**307-309.

55. **Golnar AJ, Turell MJ, LaBeaud AD, Kading RC, Hamer GL.** 2014. Predicting the mosquito species and vertebrate species involved in the theoretical transmission of Rift Valley fever virus in the United States. PLoS Negl Trop Dis **8:**e3163.

56. **Scott GR, Coackley W, Roach RW, Cowdy NR.** 1963. Rift Valley fever in camels. J Pathol Bacteriol **86:**229-231.

57. **Anderson GW, Jr., Slone TW, Jr., Peters CJ.** 1987. Pathogenesis of Rift Valley fever virus (RVFV) in inbred rats. Microb Pathog **2:**283-293.

58. **Easterday B, Murphy, LC, Bennet ,DG.** 1962. Experimental Rift Valley fever in calves, goats, and pigs. Am J Vet Res **23:**1224-1230.

59. **Swanepoel R and Coetzer JAW.** 2004. Rift Valley fever. *In* Infectious diseases of livestock with special reference to Souther Africa. Oxford University Press. **2**: 1037-1070.

60. **Olive MM, Goodman SM, Reynes JM.** 2012. The role of wild mammals in the maintenance of Rift Valley fever virus. J Wildl Dis **48:**241-266.

61. **Anderson EC, Rowe LW.** 1998. The prevalence of antibody to the viruses of bovine virus diarrhoea, bovine herpes virus 1, rift valley fever, ephemeral fever and bluetongue and to Leptospira sp in free-ranging wildlife in Zimbabwe. Epidemiol Infect **121:**441-449.

62. **Evans A, Gakuya F, Paweska JT, Rostal M, Akoolo L, Van Vuren PJ, Manyibe T, Macharia JM, Ksiazek TG, Feikin DR, Breiman RF, Kariuki Njenga M.** 2008. Prevalence of antibodies against Rift Valley fever virus in Kenyan wildlife. Epidemiol Infect **136:**1261-1269.

63. **Gaudreault NN, Indran SV, Bryant PK, Richt JA, Wilson WC.** 2015. Comparison of Rift Valley fever virus replication in North American livestock and wildlife cell lines. Front Microbiol **6:**664.

64. **Van der Lugt JJ, Coetzer JA, Smit MM.** 1996. Distribution of viral antigen in tissues of new-born lambs infected with Rift Valley fever virus. Onderstepoort J Vet Res **63:**341-347.

65. **Easterday B, Murphy L, Bennet D.** 1962. Experimental rift valley fever in lambs and sheeps. Am J Vet Res **23:**1231-1240.

66. **Weingartl HM, Miller M, Nfon C, Wilson WC.** 2014. Development of a Rift Valley fever virus viremia challenge model in sheep and goats. Vaccine **32:**2337-2344.

67. **Nfon CK, Marszal P, Zhang S, Weingartl HM.** 2012. Innate immune response to Rift Valley fever virus in goats. PLoS Negl Trop Dis **6:**e1623.

68. **Odendaal L, Fosgate GT, Romito M, Coetzer JA, Clift SJ.** 2014. Sensitivity and specificity of real-time reverse transcription polymerase chain reaction, histopathology, and immunohistochemical labeling for the detection of Rift Valley fever virus in naturally infected cattle and sheep. J Vet Diagn Invest **26:**49-60.

69. **Swai ES, Sindato C.** 2015. Seroprevalence of Rift Valley fever virus infection in camels (dromedaries) in northern Tanzania. Trop Anim Health Prod **47:**347-352.

70. **El Mamy AB, Baba MO, Barry Y, Isselmou K, Dia ML, El Kory MO, Diop M, Lo MM, Thiongane Y, Bengoumi M, Puech L, Plee L, Claes F, de La Rocque S, Doumbia B.** 2011. Unexpected Rift Valley fever outbreak, northern Mauritania. Emerg Infect Dis **17:**1894-1896.

71. **Rissmann M, Ulrich R, Schroder C, Hammerschmidt B, Hanke D, Mroz C, Groschup MH, Eiden M.** 2017. Vaccination of alpacas against Rift Valley fever virus: Safety, immunogenicity and pathogenicity of MP-12 vaccine. Vaccine **35:**655-662.

72. **Smith DR, Steele KE, Shamblin J, Honko A, Johnson J, Reed C, Kennedy M, Chapman JL, Hensley LE.** 2010. The pathogenesis of Rift Valley fever virus in the mouse model. Virology **407:**256-267.

73. **Reed C, Lin K, Wilhelmsen C, Friedrich B, Nalca A, Keeney A, Donnelly G, Shamblin J, Hensley LE, Olinger G, Smith DR.** 2013. Aerosol exposure to Rift Valley fever virus causes earlier and more severe neuropathology in the murine model, which has important implications for therapeutic development. PLoS Negl Trop Dis **7:**e2156.

74. **Le Coupanec A, Babin D, Fiette L, Jouvion G, Ave P, Misse D, Bouloy M, Choumet V.** 2013. Aedes mosquito saliva modulates Rift Valley fever virus pathogenicity. PLoS Negl Trop Dis **7:**e2237.

75. **Scharton D, Van Wettere AJ, Bailey KW, Vest Z, Westover JB, Siddharthan V, Gowen BB.** 2015. Rift Valley fever virus infection in golden Syrian hamsters. PLoS One **10:**e0116722.

76. **Findlay GM, Howard EM.** 1952. The susceptibility of rats to Rift Valley fever in relation to age. Ann Trop Med Parasitol **46:**33-37.

77. **Anderson GW, Jr., Slone TW, Jr., Peters CJ.** 1988. The gerbil, Meriones unguiculatus, a model for Rift Valley fever viral encephalitis. Arch Virol **102:**187-196.

78. **Peters CJ, Jones D, Trotter R, Donaldson J, White J, Stephen E, Slone TW, Jr.** 1988. Experimental Rift Valley fever in rhesus macaques. Arch Virol **99:**31-44.

79. **Smith DR, Bird BH, Lewis B, Johnston SC, McCarthy S, Keeney A, Botto M, Donnelly G, Shamblin J, Albarino CG, Nichol ST, Hensley LE.** 2012. Development of a novel nonhuman primate model for Rift Valley fever. J Virol **86:**2109-2120.

80. **Morrill JC, Jennings GB, Johnson AJ, Cosgriff TM, Gibbs PH, Peters CJ.** 1990. Pathogenesis of Rift Valley fever in rhesus monkeys: role of interferon response. Arch Virol **110:**195-212.

81. **Findlay G.** 1932. The infectivity of Rift Valley Fever for monkeys. Trans Roy Soc Trop Med Hyg**:**161-168.

82. **Madani TA, Al-Mazrou YY, Al-Jeffri MH, Mishkhas AA, Al-Rabeah AM, Turkistani AM, Al-Sayed MO, Abodahish AA, Khan AS, Ksiazek TG, Shobokshi O.** 2003. Rift Valley fever epidemic in Saudi Arabia: epidemiological, clinical, and laboratory characteristics. Clin Infect Dis **37:**1084-1092.

83. **Al-Khuwaitir TS, Al-Moghairi AM, Sherbeeni SM, Al-Ghamdi AS.** 2004. Rift Valley fever hepatitis complicated by disseminated intravascular coagulation and hepatorenal syndrome. Saudi Med J **25:**528-531.

84. **El Imam M, El Sabiq M, Omran M, Abdalkareem A, El Gaili Mohamed MA, Elbashir A, Khalafala O.** 2009. Acute renal failure associated with the Rift Valley fever: a single center study. Saudi J Kidney Dis Transpl **20:**1047-1052.

85. **Bouloy M, Flick R.** 2009. Reverse genetics technology for Rift Valley fever virus: current and future applications for the development of therapeutics and vaccines. Antiviral Res **84:**101-118.

86. **Faburay B, LaBeaud AD, McVey DS, Wilson WC, Richt JA.** 2017. Current Status of Rift Valley Fever Vaccine Development. Vaccines (Basel) **5**.

87. **Smithburn KC.** 1949. Rift Valley fever; the neurotropic adaptation of the virus and the experimental use of this modified virus as a vaccine. Br J Exp Pathol **30:**1-16.

88. **Sall AA, Zanotto PM, Sene OK, Zeller HG, Digoutte JP, Thiongane Y, Bouloy M.** 1999. Genetic reassortment of Rift Valley fever virus in nature. J Virol **73:**8196-8200.

89. **Botros B, Omar A, Elian K, Mohamed G, Soliman A, Salib A, Salman D, Saad M, Earhart K.** 2006. Adverse response of non-indigenous cattle of European breeds to live attenuated Smithburn Rift Valley fever vaccine. J Med Virol **78:**787-791.

90. **Muller R, Saluzzo JF, Lopez N, Dreier T, Turell M, Smith J, Bouloy M.** 1995. Characterization of clone 13, a naturally attenuated avirulent isolate of Rift Valley fever virus, which is altered in the small segment. Am J Trop Med Hyg **53:**405-411.

91. **Dungu B, Louw I, Lubisi A, Hunter P, von Teichman BF, Bouloy M.** 2010. Evaluation of the efficacy and safety of the Rift Valley Fever Clone 13 vaccine in sheep. Vaccine **28:**4581-4587.

92. **von Teichman B, Engelbrecht A, Zulu G, Dungu B, Pardini A, Bouloy M.** 2011. Safety and efficacy of Rift Valley fever Smithburn and Clone 13 vaccines in calves. Vaccine **29:**5771-5777.

93. **Makoschey B, van Kilsdonk E, Hubers WR, Vrijenhoek MP, Smit M, Wichgers Schreur PJ, Kortekaas J, Moulin V.** 2016. Rift Valley Fever Vaccine Virus Clone 13 Is Able to Cross the Ovine Placental Barrier Associated with Foetal Infections, Malformations, and Stillbirths. PLoS Negl Trop Dis **10:**e0004550.

94. **FAO.** 2015. The Last Hurdles Towards Rift Valley Fever Control. Rome, Italy.

95. **Morrill JC, Carpenter L, Taylor D, Ramsburg HH, Quance J, Peters CJ.** 1991. Further evaluation of a mutagen-attenuated Rift Valley fever vaccine in sheep. Vaccine **9:**35-41.

96. **Hunter P, Erasmus BJ, Vorster JH.** 2002. Teratogenicity of a mutagenised Rift Valley fever virus (MVP 12) in sheep. Onderstepoort J Vet Res **69:**95-98.

97. **Wilson WC, Bawa B, Drolet BS, Lehiy C, Faburay B, Jasperson DC, Reister L, Gaudreault NN, Carlson J, Ma W, Morozov I, McVey DS, Richt JA.** 2014. Evaluation of lamb and calf responses to Rift Valley fever MP-12 vaccination. Vet Microbiol **172:**44-50.

98. **Lokugamage N, Ikegami T.** 2017. Genetic stability of Rift Valley fever virus MP-12 vaccine during serial passages in culture cells. NPJ Vaccines **2**.

99. **Miller MM, Bennett KE, Drolet BS, Lindsay R, Mecham JO, Reeves WK, Weingartl HM, Wilson WC.** 2015. Evaluation of the Efficacy, Potential for Vector Transmission, and Duration of Immunity of MP-12, an Attenuated Rift Valley Fever Virus Vaccine Candidate, in Sheep. Clin Vaccine Immunol **22:**930-937.

100. **Morrill JC, Laughlin RC, Lokugamage N, Wu J, Pugh R, Kanani P, Adams LG, Makino S, Peters CJ.** 2013. Immunogenicity of a recombinant Rift Valley fever MP-12-NSm deletion vaccine candidate in calves. Vaccine **31:**4988-4994.

101. **Weingartl HM, Nfon CK, Zhang S, Marszal P, Wilson WC, Morrill JC, Bettinger GE, Peters CJ.** 2014. Efficacy of a recombinant Rift Valley fever virus MP-12 with NSm deletion as a vaccine candidate in sheep. Vaccine **32:**2345-2349.

102. **Morrill JC, Laughlin RC, Lokugamage N, Pugh R, Sbrana E, Weise WJ, Adams LG, Makino S, Peters CJ.** 2013. Safety and immunogenicity of recombinant Rift Valley fever MP-12 vaccine candidates in sheep. Vaccine **31:**559-565.

103. **Lihoradova OA, Indran SV, Kalveram B, Lokugamage N, Head JA, Gong B, Tigabu B, Juelich TL, Freiberg AN, Ikegami T.** 2013. Characterization of Rift Valley fever virus MP-12 strain encoding NSs of Punta Toro virus or sandfly fever Sicilian virus. PLoS Negl Trop Dis **7:**e2181.

104. **Gowen BB, Westover JB, Sefing EJ, Bailey KW, Nishiyama S, Wandersee L, Scharton D, Jung KH, Ikegami T.** 2015. MP-12 virus containing the clone 13 deletion in the NSs gene prevents lethal disease when administered after Rift Valley fever virus infection in hamsters. Front Microbiol **6:**651.

105. **Wichgers Schreur PJ, Oreshkova N, Moormann RJ, Kortekaas J.** 2014. Creation of Rift Valley fever viruses with four-segmented genomes reveals flexibility in bunyavirus genome packaging. J Virol **88:**10883-10893.

106. **Faburay B, Lebedev M, McVey DS, Wilson W, Morozov I, Young A, Richt JA.** 2014. A glycoprotein subunit vaccine elicits a strong Rift Valley fever virus neutralizing antibody response in sheep. Vector Borne Zoonotic Dis **14:**746-756.

107. **Wallace DB, Ellis CE, Espach A, Smith SJ, Greyling RR, Viljoen GJ.** 2006. Protective immune responses induced by different recombinant vaccine regimes to Rift Valley fever. Vaccine **24:**7181-7189.

108. **Kortekaas J, de Boer SM, Kant J, Vloet RP, Antonis AF, Moormann RJ.** 2010. Rift Valley fever virus immunity provided by a paramyxovirus vaccine vector. Vaccine **28:**4394-4401.

109. **Jansen van Vuren P, Tiemessen CT, Paweska JT.** 2011. Anti-nucleocapsid protein immune responses counteract pathogenic effects of Rift Valley fever virus infection in mice. PLoS One **6:**e25027.

110. **Pittman PR, Liu CT, Cannon TL, Makuch RS, Mangiafico JA, Gibbs PH, Peters CJ.** 1999. Immunogenicity of an inactivated Rift Valley fever vaccine in humans: a 12-year experience. Vaccine **18:**181-189.

111. **Randall R, Gibbs CJ, Jr., Aulisio CG, Binn LN, Harrison VR.** 1962. The development of a formalin-killed Rift Valley fever virus vaccine for use in man. J Immunol **89:**660-671.

112. **Rusnak JM, Gibbs P, Boudreau E, Clizbe DP, Pittman P.** 2011. Immunogenicity and safety of an inactivated Rift Valley fever vaccine in a 19-year study. Vaccine **29:**3222-3229.

113. **Frank-Peterside N.** 2000. Response of laboratory staff to vaccination with an inactivated Rift Valley fever vaccine--TSI-GSD 200. Afr J Med Med Sci **29:**89-92.

114. **Lubroth J, Rweyemamu MM, Viljoen G, Diallo A, Dungu B, Amanfu W.** 2007. Veterinary vaccines and their use in developing countries. Rev Sci Tech **26:**179-201.

115. **Clem AS.** 2011. Fundamentals of vaccine immunology. J Glob Infect Dis **3:**73-78.

116. **Soi RK, Rurangirwa FR, McGuire TC, Rwambo PM, DeMartini JC, Crawford TB.** 2010. Protection of sheep against Rift Valley fever virus and sheep poxvirus with a recombinant capripoxvirus vaccine. Clin Vaccine Immunol **17:**1842-1849.

117. **Papin JF, Verardi PH, Jones LA, Monge-Navarro F, Brault AC, Holbrook MR, Worthy MN, Freiberg AN, Yilma TD.** 2011. Recombinant Rift Valley fever vaccines

induce protective levels of antibody in baboons and resistance to lethal challenge in mice. Proc Natl Acad Sci U S A **108:**14926-14931.

118. **Busquets N, Lorenzo G, Lopez-Gil E, Rivas R, Solanes D, Galindo-Cardiel I, Abad FX, Rodriguez F, Bensaid A, Warimwe G, Gilbert SC, Domingo M, Brun A.** 2014. Efficacy assessment of an MVA vectored Rift Valley Fever vaccine in lambs. Antiviral Res **108:**165-172.

119. **Heise MT, Whitmore A, Thompson J, Parsons M, Grobbelaar AA, Kemp A, Paweska JT, Madric K, White LJ, Swanepoel R, Burt FJ.** 2009. An alphavirus replicon-derived candidate vaccine against Rift Valley fever virus. Epidemiol Infect **137:**1309-1318.

120. **Gorchakov R, Volkova E, Yun N, Petrakova O, Linde NS, Paessler S, Frolova E, Frolov I.** 2007. Comparative analysis of the alphavirus-based vectors expressing Rift Valley fever virus glycoproteins. Virology **366:**212-225.

121. **Holman DH, Penn-Nicholson A, Wang D, Woraratanadharm J, Harr MK, Luo M, Maher EM, Holbrook MR, Dong JY.** 2009. A complex adenovirus-vectored vaccine against Rift Valley fever virus protects mice against lethal infection in the presence of preexisting vector immunity. Clin Vaccine Immunol **16:**1624-1632.

122. **Warimwe GM, Gesharisha J, Carr BV, Otieno S, Otingah K, Wright D, Charleston B, Okoth E, Elena LG, Lorenzo G, Ayman el B, Alharbi NK, Al-dubaib MA, Brun A, Gilbert SC, Nene V, Hill AV.** 2016. Chimpanzee Adenovirus Vaccine Provides Multispecies Protection against Rift Valley Fever. Sci Rep **6:**20617.

123. **Warimwe GM, Lorenzo G, Lopez-Gil E, Reyes-Sandoval A, Cottingham MG, Spencer AJ, Collins KA, Dicks MD, Milicic A, Lall A, Furze J, Turner AV, Hill AV,**

Brun A, Gilbert SC. 2013. Immunogenicity and efficacy of a chimpanzee adenovirus-vectored Rift Valley fever vaccine in mice. Virol J **10:**349.

124. **Said A, Elmanzalawy M, Ma G, Damiani AM, Osterrieder N.** 2017. An equine herpesvirus type 1 (EHV-1) vector expressing Rift Valley fever virus (RVFV) Gn and Gc induces neutralizing antibodies in sheep. Virol J **14:**154.

125. **Hasson SSAA, Al-Busaidi JKZ, Sallaam TA.** 2015. The past, current and future trends in DNA vaccine immunisations. Asian Pacific Journal of Tropical Biomedicine **5:**344-353.

126. **Lagerqvist N, Naslund J, Lundkvist A, Bouloy M, Ahlm C, Bucht G.** 2009. Characterisation of immune responses and protective efficacy in mice after immunisation with Rift Valley Fever virus cDNA constructs. Virol J **6:**6.

127. **Boshra H, Lorenzo G, Rodriguez F, Brun A.** 2011. A DNA vaccine encoding ubiquitinated Rift Valley fever virus nucleoprotein provides consistent immunity and protects IFNAR(-/-) mice upon lethal virus challenge. Vaccine **29:**4469-4475.

128. **Spik K, Shurtleff A, McElroy AK, Guttieri MC, Hooper JW, SchmalJohn C.** 2006. Immunogenicity of combination DNA vaccines for Rift Valley fever virus, tick-borne encephalitis virus, Hantaan virus, and Crimean Congo hemorrhagic fever virus. Vaccine **24:**4657-4666.

129. **Grgacic EV, Anderson DA.** 2006. Virus-like particles: passport to immune recognition. Methods **40:**60-65.

130. **Naslund J, Lagerqvist N, Habjan M, Lundkvist A, Evander M, Ahlm C, Weber F, Bucht G.** 2009. Vaccination with virus-like particles protects mice from lethal infection of Rift Valley Fever Virus. Virology **385:**409-415.

131. **Dodd KA, Bird BH, Metcalfe MG, Nichol ST, Albarino CG.** 2012. Single-dose immunization with virus replicon particles confers rapid robust protection against Rift Valley fever virus challenge. J Virol **86:**4204-4212.

132. **de Boer SM, Kortekaas J, Antonis AF, Kant J, van Oploo JL, Rottier PJ, Moormann RJ, Bosch BJ.** 2010. Rift Valley fever virus subunit vaccines confer complete protection against a lethal virus challenge. Vaccine **28:**2330-2339.

133. **Uttenthal A, Parida S, Rasmussen TB, Paton DJ, Haas B, Dundon WG.** 2010. Strategies for differentiating infection in vaccinated animals (DIVA) for foot-and-mouth disease, classical swine fever and avian influenza. Expert Rev Vaccines **9:**73-87.

134. **Barrett A, Stanberry L.** 2009. Vaccines for Biodefense and Emerging and Neglected Diseases. Academic Press, London, United Kingdom.

135. **Anderson GW, Jr., Saluzzo JF, Ksiazek TG, Smith JF, Ennis W, Thureen D, Peters CJ, Digoutte JP.** 1989. Comparison of in vitro and in vivo systems for propagation of Rift Valley fever virus from clinical specimens. Res Virol **140:**129-138.

136. **Filone CM, Hanna SL, Caino MC, Bambina S, Doms RW, Cherry S.** 2010. Rift valley fever virus infection of human cells and insect hosts is promoted by protein kinase C epsilon. PLoS One **5:**e15483.

137. **Mansfield KL, Banyard AC, McElhinney L, Johnson N, Horton DL, Hernandez-Triana LM, Fooks AR.** 2015. Rift Valley fever virus: A review of diagnosis and vaccination, and implications for emergence in Europe. Vaccine **33:**5520-5531.

138. **Billecocq A, Vialat P, Bouloy M.** 1996. Persistent infection of mammalian cells by Rift Valley fever virus. J Gen Virol **77 (Pt 12):**3053-3062.

139. **Coetzer JA.** 1977. The pathology of Rift Valley fever. I. Lesions occurring in natural cases in new-born lambs. Onderstepoort J Vet Res **44:**205-211.

140. **Rippy MK, Topper MJ, Mebus CA, Morrill JC.** 1992. Rift Valley fever virus-induced encephalomyelitis and hepatitis in calves. Vet Pathol **29:**495-502.

141. **Coetzer JA.** 1982. The pathology of Rift Valley fever. II. Lesions occurring in field cases in adult cattle, calves and aborted foetuses. Onderstepoort J Vet Res **49:**11-17.

142. **Shieh WJ, Paddock CD, Lederman E, Rao CY, Gould LH, Mohamed M, Mosha F, Mghamba J, Bloland P, Njenga MK, Mutonga D, Samuel AA, Guarner J, Breiman RF, Zaki SR.** 2010. Pathologic studies on suspect animal and human cases of Rift Valley fever from an outbreak in Eastern Africa, 2006-2007. Am J Trop Med Hyg **83:**38-42.

143. **Drolet BS, Weingartl HM, Jiang J, Neufeld J, Marszal P, Lindsay R, Miller MM, Czub M, Wilson WC.** 2012. Development and evaluation of one-step rRT-PCR and immunohistochemical methods for detection of Rift Valley fever virus in biosafety level 2 diagnostic laboratories. J Virol Methods **179:**373-382.

144. **Njenga MK, Paweska J, Wanjala R, Rao CY, Weiner M, Omballa V, Luman ET, Mutonga D, Sharif S, Panning M, Drosten C, Feikin DR, Breiman RF.** 2009. Using a field quantitative real-time PCR test to rapidly identify highly viremic rift valley fever cases. J Clin Microbiol **47:**1166-1171.

145. **Lutomiah J, Omondi D, Masiga D, Mutai C, Mireji PO, Ongus J, Linthicum KJ, Sang R.** 2014. Blood meal analysis and virus detection in blood-fed mosquitoes collected during the 2006-2007 Rift Valley fever outbreak in Kenya. Vector Borne Zoonotic Dis **14:**656-664.

146. **Ibrahim MS, Turell MJ, Knauert FK, Lofts RS.** 1997. Detection of Rift Valley fever virus in mosquitoes by RT-PCR. Mol Cell Probes **11:**49-53.

147. **Wilson WC, Romito M, Jasperson DC, Weingartl H, Binepal YS, Maluleke MR, Wallace DB, van Vuren PJ, Paweska JT.** 2013. Development of a Rift Valley fever real-time RT-PCR assay that can detect all three genome segments. J Virol Methods **193:**426-431.

148. **Bird BH, Bawiec DA, Ksiazek TG, Shoemaker TR, Nichol ST.** 2007. Highly sensitive and broadly reactive quantitative reverse transcription-PCR assay for high-throughput detection of Rift Valley fever virus. J Clin Microbiol **45:**3506-3513.

149. **Sall AA, Macondo EA, Sene OK, Diagne M, Sylla R, Mondo M, Girault L, Marrama L, Spiegel A, Diallo M, Bouloy M, Mathiot C.** 2002. Use of reverse transcriptase PCR in early diagnosis of Rift Valley fever. Clin Diagn Lab Immunol **9:**713-715.

150. **Garcia S, Crance JM, Billecocq A, Peinnequin A, Jouan A, Bouloy M, Garin D.** 2001. Quantitative real-time PCR detection of Rift Valley fever virus and its application to evaluation of antiviral compounds. J Clin Microbiol **39:**4456-4461.

151. **Drosten C, Gottig S, Schilling S, Asper M, Panning M, Schmitz H, Gunther S.** 2002. Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, dengue virus, and yellow fever virus by real-time reverse transcription-PCR. J Clin Microbiol **40:**2323-2330.

152. **Das S, Rundell MS, Mirza AH, Pingle MR, Shigyo K, Garrison AR, Paragas J, Smith SK, Olson VA, Larone DH, Spitzer ED, Barany F, Golightly LM.** 2015. A

Multiplex PCR/LDR Assay for the Simultaneous Identification of Category A Infectious Pathogens: Agents of Viral Hemorrhagic Fever and Variola Virus. PLoS One **10:**e0138484.

153. **Yeh JY, Lee JH, Seo HJ, Park JY, Moon JS, Cho IS, Choi IS, Park SY, Song CS, Lee JB.** 2011. Simultaneous detection of Rift Valley Fever, bluetongue, rinderpest, and Peste des petits ruminants viruses by a single-tube multiplex reverse transcriptase-PCR assay using a dual-priming oligonucleotide system. J Clin Microbiol **49:**1389-1394.

154. **Venter M, Zaayman D, van Niekerk S, Stivaktas V, Goolab S, Weyer J, Paweska JT, Swanepoel R.** 2014. Macroarray assay for differential diagnosis of meningoencephalitis in southern Africa. J Clin Virol **60:**50-56.

155. **Le Roux CA, Kubo T, Grobbelaar AA, van Vuren PJ, Weyer J, Nel LH, Swanepoel R, Morita K, Paweska JT.** 2009. Development and evaluation of a real-time reverse transcription-loop-mediated isothermal amplification assay for rapid detection of Rift Valley fever virus in clinical specimens. J Clin Microbiol **47:**645-651.

156. **Peyrefitte CN, Boubis L, Coudrier D, Bouloy M, Grandadam M, Tolou HJ, Plumet S.** 2008. Real-time reverse-transcription loop-mediated isothermal amplification for rapid detection of rift valley Fever virus. J Clin Microbiol **46:**3653-3659.

157. **Euler M, Wang Y, Nentwich O, Piepenburg O, Hufert FT, Weidmann M.** 2012. Recombinase polymerase amplification assay for rapid detection of Rift Valley fever virus. J Clin Virol **54:**308-312.

158. **Mansuroglu Z, Josse T, Gilleron J, Billecocq A, Leger P, Bouloy M, Bonnefoy E.** 2010. Nonstructural NSs protein of rift valley fever virus interacts with pericentromeric

DNA sequences of the host cell, inducing chromosome cohesion and segregation defects. J Virol **84:**928-939.

159. **Copeland AM, Van Deusen NM, Schmaljohn CS.** 2015. Rift Valley fever virus NSS gene expression correlates with a defect in nuclear mRNA export. Virology **486:**88-93.

160. **Wichgers Schreur PJ, Kortekaas J.** 2016. Single-Molecule FISH Reveals Non-selective Packaging of Rift Valley Fever Virus Genome Segments. PLoS Pathog **12:**e1005800.

161. **Swanepoel R, Struthers JK, Erasmus MJ, Shepherd SP, McGillivray GM, Erasmus BJ, Barnard BJ.** 1986. Comparison of techniques for demonstrating antibodies to Rift Valley fever virus. J Hyg (Lond) **97:**317-329.

162. **Earley E, Peralta PH, Johnson KM.** 1967. A plaque neutralization method for arboviruses. Proc Soc Exp Biol Med **125:**741-747.

163. **Paweska JT, Burt FJ, Anthony F, Smith SJ, Grobbelaar AA, Croft JE, Ksiazek TG, Swanepoel R.** 2003. IgG-sandwich and IgM-capture enzyme-linked immunosorbent assay for the detection of antibody to Rift Valley fever virus in domestic ruminants. J Virol Methods **113:**103-112.

164. **Williams R, Ellis CE, Smith SJ, Potgieter CA, Wallace D, Mareledwane VE, Majiwa PA.** 2011. Validation of an IgM antibody capture ELISA based on a recombinant nucleoprotein for identification of domestic ruminants infected with Rift Valley fever virus. J Virol Methods **177:**140-146.

165. **Jansen van Vuren P, Potgieter AC, Paweska JT, van Dijk AA.** 2007. Preparation and evaluation of a recombinant Rift Valley fever virus N protein for the detection of IgG and IgM antibodies in humans and animals by indirect ELISA. J Virol Methods **140:**106-114.

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

167. **Paweska JT, Mortimer E, Leman PA, Swanepoel R.** 2005. An inhibition enzyme-linked immunosorbent assay for the detection of antibody to Rift Valley fever virus in humans, domestic and wild ruminants. J Virol Methods **127:**10-18.

168. **Paweska JT, Burt FJ, Swanepoel R.** 2005. Validation of IgG-sandwich and IgM-capture ELISA for the detection of antibody to Rift Valley fever virus in humans. J Virol Methods **124:**173-181.

169. **Niklasson B, Peters CJ, Grandien M, Wood O.** 1984. Detection of human immunoglobulins G and M antibodies to Rift Valley fever virus by enzyme-linked immunosorbent assay. J Clin Microbiol **19:**225-229.

170. **Paweska JT, Jansen van Vuren P, Swanepoel R.** 2007. Validation of an indirect ELISA based on a recombinant nucleocapsid protein of Rift Valley fever virus for the detection of IgG antibody in humans. J Virol Methods **146:**119-124.

171. **Paweska JT, van Vuren PJ, Kemp A, Buss P, Bengis RG, Gakuya F, Breiman RF, Njenga MK, Swanepoel R.** 2008. Recombinant nucleocapsid-based ELISA for detection of IgG antibody to Rift Valley fever virus in African buffalo. Vet Microbiol **127:**21-28.

172. **Kim HJ, Nah JJ, Moon JS, Ko YJ, Yoo HS, Kweon CH.** 2012. Competitive ELISA for the detection of antibodies to Rift Valley fever virus in goats and cattle. J Vet Med Sci **74:**321-327.

173. **Jäckel S, Eiden M, Balkema-Buschmann A, Ziller M, van Vuren PJ, Paweska JT, Groschup MH.** 2013. A novel indirect ELISA based on glycoprotein Gn for the

detection of IgG antibodies against Rift Valley fever virus in small ruminants. Res Vet Sci **95:**725-730.

174. **Faburay B, Wilson W, McVey DS, Drolet BS, Weingartl H, Madden D, Young A, Ma W, Richt JA.** 2013. Rift Valley fever virus structural and nonstructural proteins: recombinant protein expression and immunoreactivity against antisera from sheep. Vector Borne Zoonotic Dis **13:**619-629.

175. **McElroy AK, Albariño CG, Nichol ST.** 2009. Development of a RVFV ELISA that can distinguish infected from vaccinated animals. Virol J **6:**125.

176. **Fernandez JC, Billecocq A, Durand JP, Cetre-Sossah C, Cardinale E, Marianneau P, Pepin M, Tordo N, Bouloy M.** 2012. The nonstructural protein NSs induces a variable antibody response in domestic ruminants naturally infected with Rift Valley fever virus. Clin Vaccine Immunol **19:**5-10.

177. **Faburay B, Wilson WC, Gaudreault NN, Davis AS, Shivanna V, Bawa B, Sunwoo SY, Ma W, Drolet BS, Morozov I, McVey DS, Richt JA.** 2016. A Recombinant Rift Valley Fever Virus Glycoprotein Subunit Vaccine Confers Full Protection against Rift Valley Fever Challenge in Sheep. Sci Rep **6:**27719.

178. **WHO.** 2017. 2017 Annual review of diseases prioritized under the Research and Development Blueprint. http://www.who.int/blueprint/what/research-development/2017-Prioritization-Long-Report.pdf?ua=1. Accessed 01/27/18.

179. **CDC.** 2017. Guidance on the Transfer of Select Agents and Toxins. https://www.selectagents.gov/tgd-intro.html. Accessed 02/17/18.

180. **van der Wal FJ, Achterberg RP, de Boer SM, Boshra H, Brun A, Maassen CB, Kortekaas J.** 2012. Bead-based suspension array for simultaneous detection of

antibodies against the Rift Valley fever virus nucleocapsid and Gn glycoprotein. J Virol Methods **183:**99-105.

181. **Odendaal L, Clift S, Fosgate G, Davis AS.** 2018. Tissue tropism and target cells of natural Rift Valley fever virus infection in adult sheep. Manuscript submitted for peer review.

182. **Jackel S, Eiden M, Dauber M, Balkema-Buschmann A, Brun A, Groschup MH.** 2014. Generation and application of monoclonal antibodies against Rift Valley fever virus nucleocapsid protein NP and glycoproteins Gn and Gc. Arch Virol **159:**535-546.

183. **Martin-Folgar R, Lorenzo G, Boshra H, Iglesias J, Mateos F, Borrego B, Brun A.** 2010. Development and characterization of monoclonal antibodies against Rift Valley fever virus nucleocapsid protein generated by DNA immunization. MAbs **2:**275-284.

184. **Zaki A, Coudrier D, Yousef AI, Fakeeh M, Bouloy M, Billecocq A.** 2006. Production of monoclonal antibodies against Rift Valley fever virus Application for rapid diagnosis tests (virus detection and ELISA) in human sera. J Virol Methods **131:**34-40.

185. **Caliendo AM, Gilbert DN, Ginocchio CC, Hanson KE, May L, Quinn TC, Tenover FC, Alland D, Blaschke AJ, Bonomo RA, Carroll KC, Ferraro MJ, Hirschhorn LR, Joseph WP, Karchmer T, MacIntyre AT, Reller LB, Jackson AF, Infectious Diseases Society of A.** 2013. Better tests, better care: improved diagnostics for infectious diseases. Clin Infect Dis **57 Suppl 3:**S139-170.

186. **Bacarese-Hamilton T, Mezzasoma L, Ardizzoni A, Bistoni F, Crisanti A.** 2004. Serodiagnosis of infectious diseases with antigen microarrays. J Appl Microbiol **96:**10-17.

187. **Zhong L, Zhang W, Zer C, Ge K, Gao X, Kernstine KH.** 2010. Protein microarray: sensitive and effective immunodetection for drug residues. BMC Biotechnol **10:**12.

188. **Ellington AA, Kullo IJ, Bailey KR, Klee GG.** 2010. Antibody-based protein multiplex platforms: technical and operational challenges. Clin Chem **56:**186-193.

189. **Sjoberg R, Mattsson C, Andersson E, Hellstrom C, Uhlen M, Schwenk JM, Ayoglu B, Nilsson P.** 2016. Exploration of high-density protein microarrays for antibody validation and autoimmunity profiling. N Biotechnol **33:**582-592.

190. **Tighe PJ, Ryder RR, Todd I, Fairclough LC.** 2015. ELISA in the multiplex era: potentials and pitfalls. Proteomics Clin Appl **9:**406-422.

191. **Joos T, Bachmann J.** 2009. Protein microarrays: potentials and limitations. Front Biosci (Landmark Ed) **14:**4376-4385.

192. **Horan PK, Wheeless LL, Jr.** 1977. Quantitative single cell analysis and sorting. Science **198:**149-157.

193. **Nolan JP, Mandy F.** 2006. Multiplexed and microparticle-based analyses: quantitative tools for the large-scale analysis of biological systems. Cytometry A **69:**318-325.

194. **Christopher-Hennings J, Araujo KP, Souza CJ, Fang Y, Lawson S, Nelson EA, Clement T, Dunn M, Lunney JK.** 2013. Opportunities for bead-based multiplex assays in veterinary diagnostic laboratories. J Vet Diagn Invest **25:**671-691.

195. **Luminex.** 2018. xMAP Technology. https://www.luminexcorp.com/research/our-technology/xmap-technology/. Accessed 02/06/2018.

196. **Moncunill G, Aponte JJ, Nhabomba AJ, Dobano C.** 2013. Performance of multiplex commercial kits to quantify cytokine and chemokine responses in culture supernatants from Plasmodium falciparum stimulations. PLoS One **8:**e52587.

197. **Reslova N, Michna V, Kasny M, Mikel P, Kralik P.** 2017. xMAP Technology: Applications in Detection of Pathogens. Front Microbiol **8:**55.

198. **Hossain MM, Wilson WC, Faburay B, Richt J, McVey DS, Rowland RR.** 2016. Multiplex Detection of IgG and IgM to Rift Valley Fever Virus Nucleoprotein, Nonstructural Proteins, and Glycoprotein in Ovine and Bovine. Vector Borne Zoonotic Dis **16:**550-557.

199. **Li ZN, Trost JF, Weber KM, LeMasters EH, Nasreen S, Esfandiari J, Gunasekera AH, McCausland M, Sturm-Ramirez K, Wrammert J, Gregory S, Veguilla V, Stevens J, Miller JD, Katz JM, Levine MZ.** 2017. Novel multiplex assay platforms to detect influenza A hemagglutinin subtype-specific antibody responses for high-throughput and in-field applications. Influenza Other Respir Viruses **11:**289-297.

200. **Satterly NG, Voorhees MA, Ames AD, Schoepp RJ.** 2017. Comparison of MagPix Assays and Enzyme-Linked Immunosorbent Assay for Detection of Hemorrhagic Fever Viruses. J Clin Microbiol **55:**68-78.

201. **Caplen H, Peters CJ, Bishop DH.** 1985. Mutagen-directed attenuation of Rift Valley fever virus as a method for vaccine development. J Gen Virol **66 (Pt 10):**2271-2277.

202. **Wichgers Schreur PJ, van Keulen L, Kant J, Kortekaas J.** 2017. Four-segmented Rift Valley fever virus-based vaccines can be applied safely in ewes during pregnancy. Vaccine **35:**3123-3128.

203. **Balasuriya UB, Shi PY, Wong SJ, Demarest VL, Gardner IA, Hullinger PJ, Ferraro GL, Boone JD, De Cino CL, Glaser AL, Renshaw RW, Ledizet M, Koski RA, MacLachlan NJ.** 2006. Detection of antibodies to West Nile virus in equine sera using microsphere immunoassay. J Vet Diagn Invest **18:**392-395.

204. **Clavijo A, Hole K, Li M, Collignon B.** 2006. Simultaneous detection of antibodies to foot-and-mouth disease non-structural proteins 3ABC, 3D, 3A and 3B by a multiplexed Luminex assay to differentiate infected from vaccinated cattle. Vaccine **24:**1693-1704.

205. **Watson DS, Reddy SM, Brahmakshatriya V, Lupiani B.** 2009. A multiplexed immunoassay for detection of antibodies against avian influenza virus. J Immunol Methods **340:**123-131.

206. **Faburay B, Gaudreault NN, Liu Q, Davis AS, Shivanna V, Sunwoo SY, Lang Y, Morozov I, Ruder M, Drolet B, Scott McVey D, Ma W, Wilson W, Richt JA.** 2016. Development of a sheep challenge model for Rift Valley fever. Virology **489:**128-140.

207. **Wilson WC, Davis AS, Gaudreault NN, Faburay B, Trujillo JD, Shivanna V, Sunwoo SY, Balogh A, Endalew A, Ma W, Drolet BS, Ruder MG, Morozov I, McVey DS, Richt JA.** 2016. Experimental Infection of Calves by Two Genetically-Distinct Strains of Rift Valley Fever Virus. Viruses **8**.

208. **Catanzariti AM, Soboleva TA, Jans DA, Board PG, Baker RT.** 2004. An efficient system for high-level expression and easy purification of authentic recombinant proteins. Protein Sci **13:**1331-1339.

209. **Giménez-Lirola LG, Mur L, Rivera B, Mogler M, Sun Y, Lizano S, Goodell C, Harris DL, Rowland RR, Gallardo C, Sánchez-Vizcaíno JM, Zimmerman J.** 2016. Detection of African Swine Fever Virus Antibodies in Serum and Oral Fluid Specimens Using a Recombinant Protein 30 (p30) Dual Matrix Indirect ELISA. PLoS One **11:**e0161230.

210. **Greiner M, Pfeiffer D, Smith RD.** 2000. Principles and practical application of the receiver-operating characteristic analysis for diagnostic tests. Prev Vet Med **45:**23-41.

211. **Lupiani B, Mozisek B, Mason PW, Lamichhane C, Reddy SM.** 2010. Simultaneous detection of avian influenza virus NP and H5 antibodies in chicken sera using a fluorescence microsphere immunoassay. Avian Dis **54:**668-672.

212. **Chen TH, Lee F, Lin YL, Pan CH, Shih CN, Lee MC, Tsai HJ.** 2013. Development of a Luminex assay for the detection of swine antibodies to non-structural proteins of foot-and-mouth disease virus. J Immunol Methods **396:**87-95.

213. **Pinette MM, Rodriguez-Lecompte JC, Pasick J, Ojkic D, Leith M, Suderman M, Berhane Y.** 2014. Development of a duplex Fluorescent Microsphere Immunoassay (FMIA) for the detection of antibody responses to influenza A and newcastle disease viruses. J Immunol Methods **405:**167-177.

214. **Deregt D, Furukawa-Stoffer TL, Tokaryk KL, Pasick J, Hughes KM, Hooper-McGrevy K, Baxi S, Baxi MK.** 2006. A microsphere immunoassay for detection of antibodies to avian influenza virus. J Virol Methods **137:**88-94.

215. **Jansen van Vuren P, Paweska JT.** 2009. Laboratory safe detection of nucleocapsid protein of Rift Valley fever virus in human and animal specimens by a sandwich ELISA. J Virol Methods **157:**15-24.

216. **Jackel S, Eiden M, Balkema-Buschmann A, Ziller M, van Vuren PJ, Paweska JT, Groschup MH.** 2013. A novel indirect ELISA based on glycoprotein Gn for the detection of IgG antibodies against Rift Valley fever virus in small ruminants. Res Vet Sci **95:**725-730.

217. **Gori A, Peri C, Quilici G, Nithichanon A, Gaudesi D, Longhi R, Gourlay L, Bolognesi M, Lertmemongkolchai G, Musco G, Colombo G.** 2016. Flexible vs Rigid Epitope Conformations for Diagnostic- and Vaccine-Oriented Applications: Novel

Insights from the Burkholderia pseudomallei BPSL2765 Pal3 Epitope. ACS Infect Dis **2:**221-230.

218. **Rizwan M, Ronnberg B, Cistjakovs M, Lundkvist A, Pipkorn R, Blomberg J.** 2016. Serology in the Digital Age: Using Long Synthetic Peptides Created from Nucleic Acid Sequences as Antigens in Microarrays. Microarrays (Basel) **5**.

219. **Gowen BB, Bailey KW, Scharton D, Vest Z, Westover JB, Skirpstunas R, Ikegami T.** 2013. Post-exposure vaccination with MP-12 lacking NSs protects mice against lethal Rift Valley fever virus challenge. Antiviral Res **98:**135-143.

220. **van der Wal FJ, Jelsma T, Fijten H, Achterberg RP, Loeffen WL.** 2016. Towards a peptide-based suspension array for the detection of pestivirus antibodies in swine. J Virol Methods **235:**15-20.

221. **Gall JG, Pardue ML.** 1969. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. Proc Natl Acad Sci U S A **63:**378-383.

222. **McNicol AM, Farquharson MA.** 1997. In situ hybridization and its diagnostic applications in pathology. J Pathol **182:**250-261.

223. **Lawrence JB, Singer RH.** 1985. Quantitative analysis of in situ hybridization methods for the detection of actin gene expression. Nucleic Acids Res **13:**1777-1799.

224. **Levsky JM, Singer RH.** 2003. Fluorescence in situ hybridization: past, present and future. J Cell Sci **116:**2833-2838.

225. **Gaspar I, Ephrussi A.** 2015. Strength in numbers: quantitative single-molecule RNA detection assays. Wiley Interdiscip Rev Dev Biol **4:**135-150.

226. **Wilcox J.** 2000. Overview of In Situ Hybridization Methodology, Histochemical Society Annual Meeting, York, England, Sept. 3-8, 2000.

227. **Cassidy A, Jones J.** 2014. Developments in in situ hybridisation. Methods **70:**39-45.

228. **Wang F, Flanagan J, Su N, Wang LC, Bui S, Nielson A, Wu X, Vo HT, Ma XJ, Luo Y.** 2012. RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. J Mol Diagn **14:**22-29.

229. **Menezes RC, Figueiredo FB, Wise AG, Madeira MF, Oliveira RV, Schubach TM, Kiupel M, Langohr IM.** 2013. Sensitivity and specificity of in situ hybridization for diagnosis of cutaneous infection by Leishmania infantum in dogs. J Clin Microbiol **51:**206-211.

230. **Singhi AD, Westra WH.** 2010. Comparison of human papillomavirus in situ hybridization and p16 immunohistochemistry in the detection of human papillomavirus-associated head and neck cancer based on a prospective clinical experience. Cancer **116:**2166-2173.

231. **Kokkat TJ, Patel MS, McGarvey D, LiVolsi VA, Baloch ZW.** 2013. Archived formalin-fixed paraffin-embedded (FFPE) blocks: A valuable underexploited resource for extraction of DNA, RNA, and protein. Biopreserv Biobank **11:**101-106.

232. **Sommerland C, Mehraein Y, Giersberg M, Marben K, Rieder H, Rehder H.** 2002. Formalin-Fixed and Paraffin-Embedded Tissue Sections. Springer.

233. **Gilbert MT, Haselkorn T, Bunce M, Sanchez JJ, Lucas SB, Jewell LD, Van Marck E, Worobey M.** 2007. The isolation of nucleic acids from fixed, paraffin-embedded tissues-which methods are useful when? PLoS One **2:**e537.

234. **Hankin R.** 1992. In Situ Hybridzation: Principles and Applications. Laboratory Medicine **23:**764-770.

235. **Aquino de Muro M.** 2008. Molecular Biomethods Handbook, 2 ed. Humana Press.

236. **Carter BS, Fletcher JS, Thompson RC.** 2010. Analysis of messenger RNA expression by in situ hybridization using RNA probes synthesized via in vitro transcription. Methods **52:**322-331.

237. **Morris RG, Arends MJ, Bishop PE, Sizer K, Duvall E, Bird CC.** 1990. Sensitivity of digoxigenin and biotin labelled probes for detection of human papillomavirus by in situ hybridisation. J Clin Pathol **43:**800-805.

238. **Gupta D, Middleton LP, Whitaker MJ, Abrams J.** 2003. Comparison of fluorescence and chromogenic in situ hybridization for detection of HER-2/neu oncogene in breast cancer. Am J Clin Pathol **119:**381-387.

239. **Schumacher JA, Zhao EJ, Kofron MJ, Sumanas S.** 2014. Two-color fluorescent in situ hybridization using chromogenic substrates in zebrafish. Biotechniques **57:**254-256.

240. **Wiegant J, Ried T, Nederlof PM, van der Ploeg M, Tanke HJ, Raap AK.** 1991. In situ hybridization with fluoresceinated DNA. Nucleic Acids Res **19:**3237-3241.

241. **Raap AK.** 1998. Advances in fluorescence in situ hybridization. Mutat Res **400:**287-298.

242. **Bauman JG, Wiegant J, Borst P, van Duijn P.** 1980. A new method for fluorescence microscopical localization of specific DNA sequences by in situ hybridization of fluorochromelabelled RNA. Exp Cell Res **128:**485-490.

243. **Orjalo AV, Jr., Johansson HE, Ruth JL.** 2011. Stellaris fluorescence in situ hybridization (FISH) probes: a powerful tool for mRNA detection. Nature Methods.

244. **Shah J, Mark O, Weltman H, Barcelo N, Lo W, Wronska D, Kakkilaya S, Rao A, Bhat ST, Sinha R, Omar S, O'Bare P, Moro M, Gilman RH, Harris N.** 2015. Fluorescence In Situ Hybridization (FISH) Assays for Diagnosing Malaria in Endemic Areas. PLoS One **10:**e0136726.

245. **Morrison LE, Ramakrishnan R, Ruffalo TM, Wilber KA.** 2002. Labeling fluorescence in situ hybridization probes for genomic targets. Methods Mol Biol **204:**21-40.

246. **Hoshino T, Yilmaz LS, Noguera DR, Daims H, Wagner M.** 2008. Quantification of target molecules needed to detect microorganisms by fluorescence in situ hybridization (FISH) and catalyzed reporter deposition-FISH. Appl Environ Microbiol **74:**5068-5077.

247. **Deng R, Zhang K, Sun Y, Ren X, Li J.** 2017. Highly specific imaging of mRNA in single cells by target RNA-initiated rolling circle amplification. Chem Sci **8:**3668-3675.

248. **Urbanek MO, Nawrocka AU, Krzyzosiak WJ.** 2015. Small RNA Detection by in Situ Hybridization Methods. Int J Mol Sci **16:**13259-13286.

249. **Jonstrup SP, Koch J, Kjems J.** 2006. A microRNA detection system based on padlock probes and rolling circle amplification. RNA **12:**1747-1752.

250. **Ge J, Zhang LL, Liu SJ, Yu RQ, Chu X.** 2014. A highly sensitive target-primed rolling circle amplification (TPRCA) method for fluorescent in situ hybridization detection of microRNA in tumor cells. Anal Chem **86:**1808-1815.

251. **Zhang Q, Xu L, Zhang Y, Wang T, Zou X, Zhu Y, Zhao Y, Li C, Chen K, Sun Y, Sun J, Zhao Q, Wang Q.** 2017. A novel ViewRNA in situ hybridization method for the detection of the dynamic distribution of Classical Swine Fever Virus RNA in PK15 cells. Virol J **14:**81.

252. **Calabrese D, Wieland SF.** 2017. Highly Sensitive Detection of HBV RNA in Liver Tissue by In Situ Hybridization. Methods Mol Biol **1540:**119-134.

253. **Anderson CM, Zhang B, Miller M, Butko E, Wu X, Laver T, Kernag C, Kim J, Luo Y, Lamparski H, Park E, Su N, Ma XJ.** 2016. Fully Automated RNAscope In Situ

Hybridization Assays for Formalin-Fixed Paraffin-Embedded Cells and Tissues. J Cell Biochem **117:**2201-2208.

254. **Chugh P, Dittmer DP.** 2012. Potential pitfalls in microRNA profiling. Wiley Interdiscip Rev RNA **3:**601-616.

255. **Simeonov A, Nikiforov TT.** 2002. Single nucleotide polymorphism genotyping using short, fluorescently labeled locked nucleic acid (LNA) probes and fluorescence polarization detection. Nucleic Acids Res **30:**e91.

256. **Wilson WC, Weingartl HM, Drolet BS, Davé K, Harpster MH, Johnson PA, Faburay B, Ruder MG, Richt JA, McVey DS.** 2013. Diagnostic approaches for Rift Valley fever. Dev Biol (Basel) **135:**73-78.

257. **Moller L, Schunadel L, Nitsche A, Schwebke I, Hanisch M, Laue M.** 2015. Evaluation of virus inactivation by formaldehyde to enhance biosafety of diagnostic electron microscopy. Viruses **7:**666-679.

258. **Carossino M, Loynachan AT, James MacLachlan N, Drew C, Shuck KM, Timoney PJ, Del Piero F, Balasuriya UB.** 2016. Detection of equine arteritis virus by two chromogenic RNA in situ hybridization assays (conventional and RNAscope((R))) and assessment of their performance in tissues from aborted equine fetuses. Arch Virol **161:**3125-3136.

259. **Deleage C, Wietgrefe SW, Del Prete G, Morcock DR, Hao XP, Piatak M, Jr., Bess J, Anderson JL, Perkey KE, Reilly C, McCune JM, Haase AT, Lifson JD, Schacker TW, Estes JD.** 2016. Defining HIV and SIV Reservoirs in Lymphoid Tissues. Pathog Immun **1:**68-106.

260. **Fukazawa Y, Lum R, Okoye AA, Park H, Matsuda K, Bae JY, Hagen SI, Shoemaker R, Deleage C, Lucero C, Morcock D, Swanson T, Legasse AW, Axthelm MK, Hesselgesser J, Geleziunas R, Hirsch VM, Edlefsen PT, Piatak M, Estes JD, Lifson JD, Picker LJ.** 2015. B cell follicle sanctuary permits persistent productive simian immunodeficiency virus infection in elite controllers. Nat Med **21:**132-139.

261. **Lamers SL, Rose R, Ndhlovu LC, Nolan DJ, Salemi M, Maidji E, Stoddart CA, McGrath MS.** 2016. The meningeal lymphatic system: a route for HIV brain migration? Journal of NeuroVirology **22:**275-281.

262. **Resende TP, Marthaler DG, Vannucci FA.** 2017. A novel RNA-based in situ hybridization to detect Seneca Valley virus in neonatal piglets and sows affected with vesicular disease. PLoS One **12:**e0173190.

263. **Balogh A.** 2017. Virulence Characterization of Rift Valley Fever Virus Strains and Efficacy of Glycoproteins Subunit Vaccines in Mice. Masters thesis. Kansas State University College of Veterinary Medicine.

264. **Lang Y, Henningson J, Jasperson D, Li Y, Lee J, Ma J, Li Y, Cao N, Liu H, Wilson W, Richt J, Ruder M, McVey S, Ma W.** 2016. Mouse model for the Rift Valley fever virus MP12 strain infection. Vet Microbiol **195:**70-77.

265. **Palinski RM, Chen Z, Henningson JN, Lang Y, Rowland RR, Fang Y, Prickett J, Gauger PC, Hause BM.** 2016. Widespread detection and characterization of porcine parainfluenza virus 1 in pigs in the USA. J Gen Virol **97:**281-286.

266. **Lamers SL, Rose R, Ndhlovu LC, Nolan DJ, Salemi M, Maidji E, Stoddart CA, McGrath MS.** 2016. The meningeal lymphatic system: a route for HIV brain migration? J Neurovirol **22:**275-281.

267. **Carossino M, Loynachan AT, James MacLachlan N, Drew C, Shuck KM, Timoney PJ, Del Piero F, Balasuriya UB.** 2016. Detection of equine arteritis virus by two chromogenic RNA in situ hybridization assays (conventional and RNAscope(®)) and assessment of their performance in tissues from aborted equine fetuses. Arch Virol **161:**3125-3136.

268. **Gaynor AM, Zhu KW, Dela Cruz FN, Affolter VK, Pesavento PA.** 2016. Localization of Bovine Papillomavirus Nucleic Acid in Equine Sarcoids. Vet Pathol **53:**567-573.

269. **Zhu KW, Affolter VK, Gaynor AM, Dela Cruz FN, Pesavento PA.** 2015. Equine Genital Squamous Cell Carcinoma: In Situ Hybridization Identifies a Distinct Subset Containing Equus caballus Papillomavirus 2. Vet Pathol **52:**1067-1072.

270. **Mirghani H, Amen F, Moreau F, Guigay J, Ferchiou M, Melkane AE, Hartl DM, Lacau St Guily J.** 2014. Human papilloma virus testing in oropharyngeal squamous cell carcinoma: what the clinician should know. Oral Oncol **50:**1-9.

271. **Wichgers Schreur PJ, Kant J, van Keulen L, Moormann RJ, Kortekaas J.** 2015. Four-segmented Rift Valley fever virus induces sterile immunity in sheep after a single vaccination. Vaccine **33:**1459-1464.

272. **Upreti D, Wilson WC, Richt JA, Davis AS, Trujillo JD.** 2017. Reverse Transcriptase Real Time PCR Detection of Rift Valley Fever Virus RNA in Formalin-Fixed, Paraffin-Embedded Tissues. The FASEB Journal **31:**658.655.

273. **Vialat P, Muller R, Vu TH, Prehaud C, Bouloy M.** 1997. Mapping of the mutations present in the genome of the Rift Valley fever virus attenuated MP12 strain and their putative role in attenuation. Virus Res **52:**43-50.

274. **Wang Z, Portier BP, Gruver AM, Bui S, Wang H, Su N, Vo HT, Ma XJ, Luo Y, Budd GT, Tubbs RR.** 2013. Automated quantitative RNA in situ hybridization for resolution of equivocal and heterogeneous ERBB2 (HER2) status in invasive breast carcinoma. J Mol Diagn **15:**210-219.

275. **Stempel AJ, Morgans CW, Stout JT, Appukuttan B.** 2014. Simultaneous visualization and cell-specific confirmation of RNA and protein in the mouse retina. Mol Vis **20:**1366-1373.

276. **Grabinski TM, Kneynsberg A, Manfredsson FP, Kanaan NM.** 2015. A method for combining RNAscope in situ hybridization with immunohistochemistry in thick free-floating brain sections and primary neuronal cultures. PLoS One **10:**e0120120.

277. **Balogh A, Shivanna V, Faburay B, Davis AS, Sunwoo SY, Ma W, Morozov I, Wilson WC, Richt JA.** 2017. Evaluation of Protective Efficacies of Rift Valley Fever Virus Glycoproteins using a Mouse Model. Kansas State University College of Veterinary Medicine.

278. **Viruses ICoTo.** 2017. Virus Taxonomy: 2016 Release. https://talk.ictvonline.org/taxonomy/. Accessed 01/15/2018.

279. **Terasaki K, Makino S.** 2015. Interplay between the Virus and Host in Rift Valley Fever Pathogenesis. J Innate Immun **7:**450-458.

280. **Dodd KA, McElroy AK, Jones ME, Nichol ST, Spiropoulou CF.** 2013. Rift Valley fever virus clearance and protection from neurologic disease are dependent on CD4+ T cell and virus-specific antibody responses. J Virol **87:**6161-6171.

281. **BEI R.** Rift Valley Fever virus antibody catalog. https://www.beiresources.org/Catalog.aspx?f_instockflag=In+Stock%23~%23Discontinu

ed%23~%23Temporarily+Out+of+Stock&q=Rift%20Valley%20Fever. Accessed 02/2/2018.

282. **Wichgers Schreur PJ, van Keulen L, Kant J, Oreshkova N, Moormann RJ, Kortekaas J.** 2016. Co-housing of Rift Valley Fever Virus Infected Lambs with Immunocompetent or Immunosuppressed Lambs Does Not Result in Virus Transmission. Front Microbiol **7:**287.

283. **Mudhasani R, Kota KP, Retterer C, Tran JP, Whitehouse CA, Bavari S.** 2014. High content image-based screening of a protease inhibitor library reveals compounds broadly active against Rift Valley fever virus and other highly pathogenic RNA viruses. PLoS Negl Trop Dis **8:**e3095.

284. **Mudhasani R, Tran JP, Retterer C, Radoshitzky SR, Kota KP, Altamura LA, Smith JM, Packard BZ, Kuhn JH, Costantino J, Garrison AR, Schmaljohn CS, Huang IC, Farzan M, Bavari S.** 2013. IFITM-2 and IFITM-3 but not IFITM-1 restrict Rift Valley fever virus. J Virol **87:**8451-8464.

285. **Faburay B, Richt JA.** 2016. Short Interfering RNA Inhibits Rift Valley Fever Virus Replication and Degradation of Protein Kinase R in Human Cells. Front Microbiol **7:**1889.

286. **Filone CM, Heise M, Doms RW, Bertolotti-Ciarlet A.** 2006. Development and characterization of a Rift Valley fever virus cell-cell fusion assay using alphavirus replicon vectors. Virology **356:**155-164.

287. **Copeland AM, Altamura LA, Van Deusen NM, Schmaljohn CS.** 2013. Nuclear relocalization of polyadenylate binding protein during rift valley fever virus infection involves expression of the NSs gene. J Virol **87:**11659-11669.

288. **Voskuil J.** 2014. Commercial antibodies and their validation. F1000Res **3:**232.

289. **Norman GA, Eisenkot R.** 2017. Technology Transfer: From the Research Bench to Commercialization. JACC: Basic to Translational Science **2:**85-97.

290. **Jacobson RH.** 1996. Principles of Validation of Diagnostic Assays for Infectious Diseases. OIE Manual of Standards for Diagnostic Tests and Vaccines**:**8-15.

291. **Kelly TR, Karesh WB, Johnson CK, Gilardi KV, Anthony SJ, Goldstein T, Olson SH, Machalaba C, Consortium P, Mazet JA.** 2017. One Health proof of concept: Bringing a transdisciplinary approach to surveillance for zoonotic viruses at the human-wild animal interface. Prev Vet Med **137:**112-118.

292. **Fonkwo PN.** 2008. Pricing infectious disease. The economic and health implications of infectious diseases. EMBO Rep **9 Suppl 1:**S13-17.