Characterization and mitigation of emerging bunyaviruses

by

Victoria Brittany Ayers

B.S., 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/Pathobiology
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Abstract

Bunyavirus is a general term used to describe segmented, negative-sense, single-stranded RNA viruses that are considered important emerging pathogens which can play a significant role in veterinary and human public health. As a diverse group of mostly arthropod-borne viruses, they have recently been moved into the *Bunyavirales* order, which is the largest group of RNA viruses. Within the *Bunyavirales* order, Cache Valley virus (CVV) and Rift Valley fever virus (RVFV) play a significant role in veterinary and human morbidity and mortality. Presently, there are no vaccines available to prevent or control CVV and although there are RVFV veterinary vaccines, they have limitations. To address the limitations and gaps in knowledge for CVV and RVFV, several approaches were taken to advance our understanding of bunyavirus transmission and evaluate potential mitigation strategies. The objective of this dissertation was to identify competent mosquito vectors involved in the transmission of CVV and to evaluate candidate live-attenuated vaccines for CVV and RVFV to improve prevention, control, and mitigation strategies for emerging bunyaviruses. **The hypothesis for this dissertation is that recombinant live-attenuated candidate vaccines for CVV and RVFV are sufficiently immunogenic and attenuated in animals and unable to replicate in medically important mosquitoes in North America.**

Aim 1 determined the vector competence of medically important mosquito species in North America for the transmission of CVV. It was determined that *Culex* (*Cx.*) *tarsalis*, *Aedes* (*Ae.*) *aegypti*, and *Ae. albopictus* were susceptible to CVV and are competent for transmission of CVV in North America. These results provide a basis for how the dispersal of *Aedes* and *Culex* species mosquitoes across North America may significantly impact the transmission and ecology of CVV.

Aim 2 characterized a candidate live-attenuated vaccine (2delCVV) for CVV lacking the NSs and NSm genes. First, the immunogenicity of the live-attenuated 2delCVV candidate vaccine, lacking the expression of the two nonstructural genes (NSs and NSm) was evaluated and compared to an autogenous binary ethylenimine (BEI) inactivated CVV vaccine (BEI-CVV), in sheep. An autogenous vaccine was used for comparison because this type of vaccine can be approved and used by veterinarians when there are no vaccines commercially available. Although there was no significant difference in the neutralizing antibody titers, the 2delCVV candidate vaccine induced a slightly higher neutralizing antibody response than the autogenous vaccine on day 63 post-initial immunization. More importantly, 2delCVV elicited neutralizing antibody titers that could potentially confer protection against wild-type CVV through the duration of the study. After demonstrating attenuation of the live-attenuated 2delCVV candidate vaccine in sheep, the growth kinetics of 2delCVV in *Ae. albopictus* mosquitoes was evaluated. Ultimately, mosquitoes injected with the 2delCVV candidate vaccine had significantly lower infectivity than the mosquitoes injected with wild-type CVV, demonstrating restricted replication. These data provide a basis for further developing immunogenic vaccines for CVV and other bunyaviruses.

Aim 3 demonstrated the immunogenicity of a candidate live-attenuated RVFV vaccine in CD-1 mice. Using a similar approach as described for CVV, a reverse genetics system was utilized to create a live-attenuated candidate vaccine lacking the NSs and NSm genes (r2segMP12) and modified the three-segmented genome into a two-segmented genome. The regimen of a single immunization administered at an increasing dosage per group was included to determine the correlation of neutralizing antibodies induced by different dosages. The immune response induced by the live-attenuated vaccine candidate was then compared to the neutralizing

antibody titer produced by the conditionally licensed rMP12 parental vaccine strain. The r2segMP12 candidate vaccine at 10^5 PFU elicited a significantly higher neutralizing antibody response than the rMP12 vaccine at the same vaccination titer. The candidate vaccine, r2segMP12, was given as a booster dose at 10^5 PFU to assess if it would increase immunogenicity and produce a long-lasting neutralizing antibody response. Mice that received a single immunization of the r2segMP12 candidate vaccine at 10^5 PFU established a seroprotective neutralizing antibody response with a significantly higher immune response than those that received the rMP12 vaccine at the same titer. These results suggest that the superior immunogenicity of the r2segMP12 strain as compared to the rMP12 strain warrants its advancement in the process of vaccine development for RVFV and other bunyaviruses.

This work identified competent mosquito vectors and evaluated candidate live-attenuated vaccines for CVV and RVFV, which will aid in improving prevention, control, and mitigation strategies against emerging bunyaviruses. Results presented in this dissertation confirmed multiple North American mosquito species are competent vectors for the transmission of CVV, with the potential to contribute to the epizootic and enzootic transmission cycle of this virus. Developing a vaccine for CVV and RVFV is an important step to preventing future outbreaks, additionally, the methods to create these candidate vaccines could be a feasible approach to developing attenuated vaccine candidates for other emerging bunyaviruses.

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Approved by:

Major Professor
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Dedication

To my cat, Sushi.

Chapter 1 - Introduction

General overview of bunyaviruses

The *Bunyaviridae* family was established by the International Committee on Taxonomy of Viruses (ICTV) in 1975 (International Committee on Taxonomy of Viruses, 2022). It mainly consisted of a large group of enveloped viruses with tripartite, negative-sense, single-stranded, RNA genomes called bunyaviruses (International Committee on Taxonomy of Viruses, 2022). The term "bunyavirus" was derived from the name of the location where Bunyamwera virus (BUNV) was first isolated in Bunyamwera village, Uganda from *Aedes* mosquitoes during yellow fever surveillance (Smithburn et al., 1946). Originally, this family was divided into five genera: the *Bunyavirus* genus, transmitted mainly by mosquitoes with the prototype virus being BUNV, the *Phlebovirus* genus, mainly transmitted by sandflies with Sandfly fever virus (SFV) as the prototypic virus, the *Nairovirus* genus, transmitted predominantly by ticks carrying Crimean-Congo hemorrhagic fever virus (CCHFV), and later included, the *Tospovirus* genus, which was added in 1990 due to its transmission by several species of thrips, and the *Hantavirus* genus due to their molecular similarities (Elliott, 1997; Nichol et al., 2005). The difference between the original genera and the new addition of the *Tospovirus* genus and the *Hantavirus* genus is that tospoviruses are a group of viruses that infect plants and hantavirus transmission occurred through aerosolization of particles from small rodent secretions, whereas the others were arthropod-borne (Elliott, 1997; Lennette et al., 1988). With the discovery of new viruses, the *Bunyaviridae* family has expanded and undergone major changes (Blitvich et al., 2018).

In 2002, since all viruses within the *Bunyaviridae* family were considered bunyaviruses, the ICTV renamed the genus *Bunyavirus* to *Orthobunyavirus* in order to avoid misstatement

(Calisher, 2008; Mayo, 2002). Later, in 2016, the *Bunyaviridae* family was elevated to the level of an order called *Bunyavirales* (Blitvich et al., 2018; Briese et al., 2016). The *Bunyavirales* order is now the largest group of RNA viruses, consisting of 14 families, 60 genera, and over 450 species (International Committee on Taxonomy of Viruses, 2022). Although bunyaviruses have been renamed, the generic term is still used for this broad group of viruses.

Most bunyaviruses are arthropod-borne viruses (arboviruses) that are considered emerging and/or re-emerging pathogens with distinct geographic distribution (Blitvich et al., 2018; Elliott, 2009). The term arbovirus includes viruses that are spread by arthropod vectors including, mosquitoes, ticks, and flies, which will be discussed in depth more later in this chapter. Some arboviruses are well-known for causing devastating disease in animals including Akabane virus (AKAV) and Schmallenberg virus (SBV) (Inaba et al., 1975; OIE, 2017), whereas others such as Ngari virus (NRIV) and Oropouche virus (OROV) are pathogenic in humans (Gerrard et al., 2004; Travassos da Rosa et al., 2017). Additionally, several bunyaviruses have become important zoonotic pathogens, causing severe disease in both animals and humans, for example BUNV, Cache Valley virus (CVV) and Rift Valley fever virus (RVFV) (Anderson et al., 2015; Dutuze et al., 2018; Wilson, 1994). Although many of these viruses play a significant role in veterinary and/or human morbidity and mortality, most bunyaviruses do not have a vaccine for the prevention or control of outbreaks. To mitigate disease incidence in endemic areas and prevent new introductions, the development of new, or safer, human and agricultural vaccines is necessary (Horne & Vanlandingham, 2014).

The unprecedented spread of these arboviruses coupled with the relatively unique ability to expand to other vectors, and therefore, have wider geographic ranges, enhances the likelihood of an introduction or emergence into new areas. This dissertation focuses on two important

emerging bunyaviruses in the *Bunyavirales* order, CVV (genus *Orthobunyavirus*) and RVFV (genus *Phlebovirus*), by investigating the biology of CVV and RVFV to help develop knowledge that could be directly translated to other bunyaviruses and by assessing different strategies for rationally designed candidate live-attenuated vaccines for CVV and RVFV.

Orthobunyavirus

The *Orthobunyavirus* genus is part of the *Peribunyaviridae* family and *Bunyavirales* order. It contains at least 170 named viruses that have been divided into 20 serogroups on the basis of serological relatedness, which was determined by complement fixation, neutralization, hemagglutination-inhibition tests, and N protein sequence information (Elliott, 2014; King et al., 2012). In addition, several emerging orthobunyaviruses have been identified as reassortants, such as the OROV reassortants Jatobal and Iquitos viruses, which have been associated with human infections in Brazil and Peru, respectively (Aguilar et al., 2011; Briese et al., 2013; Saeed et al., 2001).

Orthobunyaviruses are mainly transmitted by blood feeding arthropods such as mosquitoes or *Culicoides* midges, are prevalent worldwide, and are commonly associated with central nervous system (CNS) disease in humans and other vertebrate (Edridge & van der Hoek, 2020; Elliott, 2014). Several orthobunyaviruses are considered emerging pathogens however, biological details are currently lacking for many of these viruses, making it challenging to develop mitigation strategies. For example, CVV is an orthobunyavirus that mainly causes disease in pregnant animals but was later recognized as a zoonotic pathogen causing rare but severe disease in humans.

While it is well-accepted that CVV is transmitted by multiple mosquito species in North America, most endemic vector species of CVV do not show preference for humans. Therefore, determining vector competence can provide information on the mosquito species responsible for the transmission of CVV from amplification hosts to humans. For example, *Aedes* (*Ae.*) *aegypti* and *Ae. albopictus* are the most important mosquito species responsible for arbovirus transmission to humans in urban areas (Chan et al., 2020). This makes them an important species to investigate for their involvement in transmitting arboviruses like CVV, especially when determining if CVV could become established in certain areas and regions that pose a threat to human health. Demonstrating vector competence in medically important mosquito species will also further our knowledge of which species should be targeted for vector control in the event of an outbreak.

Phlebovirus

The *Phlebovirus* genus is part of the *Phenuiviridae* family and *Bunyavirales* order. The genus *Phlebovirus* contains the sandfly fever group where most viruses are transmitted by the phlebotomine sandfly, although some are tick-borne, and RVFV is transmitted by mosquitoes (Bouloy, 2011). Phleboviruses are widely distributed throughout the world including the Middle East, Africa, Europe, North America, and South America (Plyusnin et al., 2012). However, detecting phleboviruses in human samples is challenging due to the overall diversity and genetic complexity of clinically relevant strains, as well as the high rate of recent phlebovirus discoveries and detection of novel reassortant viruses (Calisher & Calzolari, 2021; Lambert & Hughes, 2021).

Phlebovirus reassortment occurs when viral RNA segments are mixed or switch between related phlebovirus strains and are incorporated into the progeny viruses (Lambert & Hughes, 2021). This can occur when cells are coinfected by two closely related viruses (Sall et al., 1999). Reassortment causes concern because it can lead to novel strains that are more virulent, easily transmitted, and have a wider vector and/or host range (Gaudreault et al., 2019). A phlebovirus that has undergone genetic reassortment, potentially altering its biological properties, causing large outbreaks, and posing a serious threat to global human public health and the livestock industry is RVFV (Liu et al., 2017). This occurred when a patient returned from Angola with an infection of a novel reassortant between lineages E and A of RVFV (Liu et al., 2017). The reassortant strain contained the S gene segment from lineage A and the L and M segments from lineage E. Genomic reassortment between strains of RVFV has also previously been experimentally demonstrated in mammalian cell culture and live mosquitoes (Saluzzo & Smith, 1990; Turell et al., 1990).The reassortant viruses contained S and M segments from both mosquito and vertebrate hosts co-infected with Egyptian and Senegalese strains of RVFV. There was also an isolate from a patient in South America, potentially exposed to co-infection, during a needle injury, with a live-attenuated animal vaccine and wild-type virus that was a reassortant (Grobbelaar et al., 2011). The isolate was said to be a reassortant because it sorted with Smithburn neurotropic strain (SNS) vaccine virus in the M segment phylogenetic tree and with an isolate called SA54/10 in the other segments (Grobbelaar et al., 2011). The ability of RVFV to reassort in mosquitoes, vertebrate hosts, and humans raises safety concerns when using live-attenuated vaccines.

Most phlebovirus infections are asymptomatic but symptoms can include fever, malaise, and anorexia, and even progress to hemorrhagic fever, encephalitis, and/or retinal vasculitis

(Lambert & Hughes, 2021). The most well-known virus with the ability to manifest these severe symptoms is RVFV, which is also one of the most important zoonotic viruses in Africa (Tran et al., 2016). This is because RVFV causes high rates of abortions in ruminants leading to devastating economic losses in the agricultural industry (Muga et al., 2015). Rift Valley fever virus also causes large outbreaks of acute febrile and often fatal illness among humans (Bouloy & Weber, 2010; Ikegami & Makino, 2011).

Since the first outbreak described in Kenya, recurrent epidemics have killed thousands of humans (Balkhy & Memish, 2003; Bird et al., 2009; Daubney et al., 1931). Although the human mortality rate is usually low, in some outbreaks the mortality rate reached 45% (World Health Organization, 2007). Following a small sporadic outbreak of RVFV in 2008 – 2009, a widespread epidemic occurred in South Africa in 2010 and 2011 with more than 250 human cases, including 25 deaths (Archer et al., 2013). Safe and immunogenic vaccines are necessary for the prevention or containment of outbreaks however, there are no licensed vaccines commercially available for humans and there are limitations on the veterinary vaccines currently available. Part of this dissertation evaluates a live-attenuated vaccine using the RVFV mutagenized passage 12 (MP12) strain to assist in the advancement of vaccines for RVFV and other bunyaviruses.

General overview of arbovirology

Before discussing the distinct pathogenic mechanisms of the two bunyaviruses, CVV and RVFV, which are the focus of this dissertation, a brief overview of mosquito-borne viruses and virus-vector interactions follow.

> "Arthropod-borne (arbo)viruses are viruses which are maintained in nature principally, or to an important extent, through biological transmission between susceptible vertebrate hosts by hematophagous (blood-feeding) arthropods; they multiply and produce viremia in the vertebrates, multiply in the tissues of arthropods, and are transmitted to new vertebrates by the bites of arthropods after a period of extrinsic incubation (World Health Organization, 1967).

The first identification of an infectious agent being transmitted by an arthropod vector was by Sir Patrick Manson, when he demonstrated that *Culex* species mosquitoes could become infected with microfilariae during oral blood meal feeding in 1878 (Eldridge, 1992). Subsequently, Ronald Ross established a connection between the malaria parasite and mosquitoes in 1989 (Higgs, 2004). Carlos Findlay proposed a link between YFV and mosquitoes in 1881 but it was not until Drs. Reed, Agramonte, Carroll, and Lazear in 1900 that the transmission of YVF by mosquitoes was established (Finlay, 1937; Reed et al., 1900). Thus, the first known "arbovirus" was YFV.

The first arbovirus to be isolated in a nonhuman host was from the Bluetongue serogroup, isolated from pooled sheep blood collected in South Africa in 1901 – 1902 (Spreull, 1905). As virus isolation techniques became more widely used and more sophisticated in the 1930s, RVFV, West Nile virus (WNV), Japanese encephalitis virus (JEV), Eastern equine encephalitis virus (EEEV), Western equine encephalitis virus (WEEV), Venezuelan equine encephalitis virus (VEEV), and St. Louis encephalitis virus (SLEV) were isolated for the first time using either natural hosts or laboratory hosts (Calisher, 2005). There are now more than 500 recognized arboviruses worldwide, with 150 of which are known to cause human disease (Madewell, 2020).

Arthropod vectors that can play a critical role in transmitting a variety of human, animal, or zoonotic disease-causing pathogens include mosquitoes (class Insecta, order Diptera, family *Culicidae*) biting midges (class Insecta, order Diptera, family *Ceratopogonidae*), ticks (class

Arachnida, order Ixodida, families *Ixodidae* and *Aegasidae*) and others (Eldridge, 2005; Kondratieff & Black, 2005). These specific arthropod vectors are called hematophagous arthropods or blood feeding arthropods because they obtain blood from a vertebrate in a variety of ways. Hematophagous arthropods can acquire blood through simple biting with chewing-type mouthparts or by penetrating the skin with tubular sucking mouthparts that are a diameter small enough to cannulate blood vessels (Higgs et al., 2017). Additionally, if a competent arthropod vector obtains blood from a vertebrate that is infected, there is potential for the virus to replicate and be transmitted to a new host during subsequent feeding, known as biological transmission (Turell, Dohm, et al., 2010). Since there are several ways viruses can be transmitted by an arthropod vector, arbovirus transmission is discussed more in depth later in this chapter.

For an arthropod species to be competent for biological transmission of an arbovirus it must initially have the ability to exhibit a low titer threshold for midgut infection and support replication in the midgut epithelium (Higgs et al., 2005). The period of time the virus replicates and develops within the tissues of the vector is the extrinsic period, typically, the shorter the extrinsic period, the more successful the virus is at being transmitted (Christofferson & Mores, 2016). After replication, the progeny virus has to cross the basil lamina layer to disseminate into the hemolymph, and eventually infect and replicate in surrounding tissues and the salivary glands (Woodring et al., 1996). The salivary glands is the final location within the mosquito before being transmitted to a new host. However, there are various intra-arthropod obstacles to vector competence such as midgut infection and escape as well as salivary gland infection and escape barriers. Overcoming these barriers is essential for a virus to be transmissible.

It is possible to study the infection rate and growth kinetics of a virus or vaccine virus in non-susceptible mosquitoes by direct inoculation of the virus into the hemocoel (Huang et al.,

2020). A method called intrathoracic inoculation or injection is when the virus is directly injected into the mosquito's thorax to cause infection while bypassing the different barriers. Direct inoculation allows for short-term infection of the insect that would not normally be possible in nature after blood feeding (Huang et al., 2020). If the virus is unable to replicate after intrathoracic injection, the likelihood of biological transmission in nature is low. The technique of intrathoracic inoculation of a virus was used in chapter 3 to determine and compare the growth kinetics of a LAV virus and a wild-type strain.

Although the field of arbovirology is broad, there are similar goals shared by those involved in emerging bunyavirus research, which is the identification of emerging pathogens and the subsequent characterization of their basic biology, ecology, epidemiology, and pathogenesis. A major area of research within this field is vector competence and understanding the details of the virus-vector interactions. Understanding how the virus and vectors interact is critical when developing countermeasures to reduce the impact of arboviral diseases. In addition, identifying competent species for the transmission of arboviruses is particularly important for the advancement of our knowledge on the ecology and epidemiology of emerging and re-emerging arboviruses. Chapter 2 of this dissertation determined the vector competence of medically important mosquito species in North America for the transmission of CVV.

Virus-vector interactions

Mosquitoes are the most important arthropods in terms of human and veterinary health. Mosquitoes that are of medical importance belong to the *Culicidae* family, which comprises around 3,500 species and has distribution on all continents, except Antarctica (Harbach, 2013; Tandina et al., 2018). This family is distributed into the subfamilies *Culicinae* and *Anophelinae*

with mosquito vectors mainly belonging to the genera *Anopheles, Aedes,* and *Culex* (Berenger & Parola, 2017; Tandina et al., 2018).

Anopheles mosquitoes are of great importance to human health because they transmit pathogens such as malaria, filarial worms, and arboviruses (Machitani et al., 2020). The larvae of *Anopheles* have been found in fresh- or salt-water marshes, swamps, rice fields, grassy ditches, the edges of streams and rivers, and small temporary rain pools (Centers for Disease Control and Prevention, 2020c). Although *Anopheles* are an important mosquito to study because they transmit multiple arboviruses, the two genera of mosquitoes used in this dissertation are *Aedes* and *Culex* species, which are described below. The information about the species of mosquitoes used in this dissertation are mainly focused on within the United States because this is where CVV is endemic and where RVFV is at risk of being introduced.

Aedes species mosquitoes, specifically *Ae. aegypti* and *Ae. albopictus* are considered medically important mosquito species in North America that transmit zoonotic pathogens. *Ae. aegypti,* are commonly known as the yellow fever mosquito, because they are responsible for the transmission of YFV (genus *Flavivirus*). They are also the primary vector responsible for the spread of dengue virus (DENV, genus *Flavivirus*). They feed during the day, with peak activity at dawn and dusk and are recognized by black and white markings on their legs and a marking in the form of a lyre on the upper surface of its thorax, as shown in **Figure 1-1** (Clemons et al., 2010). *Ae. aegypti* are predominately found in southern and southeastern regions of the United States.

Ae. albopictus are found in the same areas as *Ae. aegypti* but are also heavily concentrated within regions extending from the Central states to the East coast (Khan et al., 2020). *Ae. albopictus* mosquitoes are referred to as the Asian tiger mosquito in which the name

"tiger mosquito" comes from its black and white colored pattern **(Figure 1-2).** This species of mosquito was introduced into the United States at the end of the eighteenth century and is now established in 866 counties of 26 states (Rai, 1991). *Ae. albopictus* are known to be aggressive day-time biting insects that are emerging as a major public health threat throughout the world. This is because of their previous role in DENV and chikungunya virus (CHKV, genus *Alphavirus*) outbreaks (Paupy et al., 2009; Rezza, 2012; Schaffner et al., 2013). Both *Aedes* species mosquitoes are known to transmit other emerging and re-emerging viruses and are difficult to eradicate making them important species to study (Porse et al., 2015).

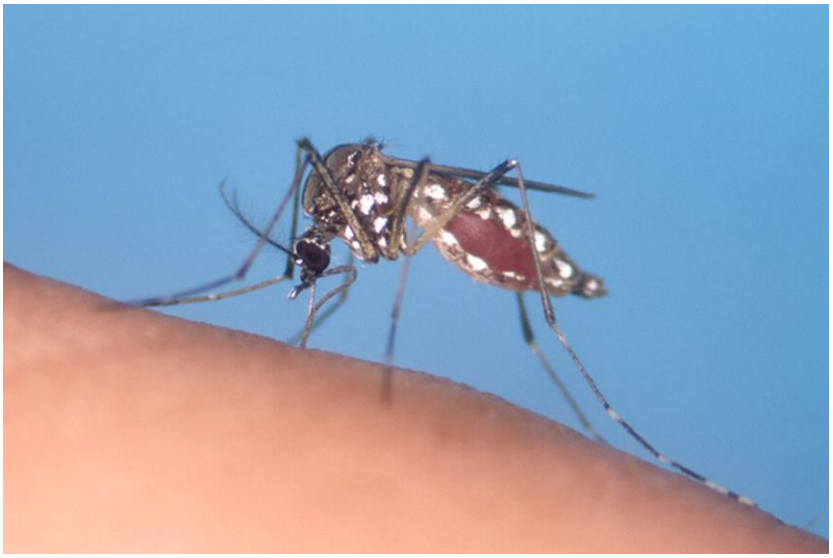

Figure 1-1. *Aedes aegypti.*
Image provided courtesy of Dr. Stephen Higgs.

Figure 1-2. *Aedes albopictus*.
Image provided courtesy of Dr. Stephen Higgs.

Other medically important mosquitoes used in this dissertation are *Cx. pipiens*, *quinquefasciatus*, and *tarsalis*, which are in the *Culex* genus. *Cx. pipiens* are referred to as the common house mosquito and is the most widely distributed mosquito worldwide (Chandra & Mukherjee, 2022). *Cx. pipiens* are medium size, brownish mosquitoes that feed on a variety of hosts. They are also major vectors of WNV (genus *Flavivirus*) and Usutu virus (USUV, *Flavivirus*) (Cook et al., 2018; Koenraadt et al., 2019).

Cx. quinquefasciatus mosquitoes are in the same subspecies complex as *Cx. pipiens* and are known as the southern house mosquito (Chandra & Mukherjee, 2022). This mosquito is small to medium in size and is identical to *Cx. pipiens* (**Figure 1-3**). *Cx. quinquefasciatus* are believed to be native to West Africa but are now found throughout subtropical and tropical areas worldwide (Samy et al., 2016). While they were originally known for spreading avian malaria

and avian pox to native Hawaiian forest birds, they are now primarily vectors of SLEV and WNV (Elias & Porch, 2022; Samy et al., 2016).

Lastly, *Cx. tarsalis* are called the Western encephalitis mosquito because it is the most important mosquito vector of arboviruses in western North America (**Figure 1-4**) (Reisen, 1993). This mosquito species is responsible for the maintenance, amplification, and epidemic transmission of SLEV (genus *Flavivirus*) and WEEV (genus *Alphavirus*) (Reisen, 1993). These three *Culex* species mosquitoes should continue to be investigated because of their highly opportunistic feeding habits and to aid in improving bunyavirus prevention measures.

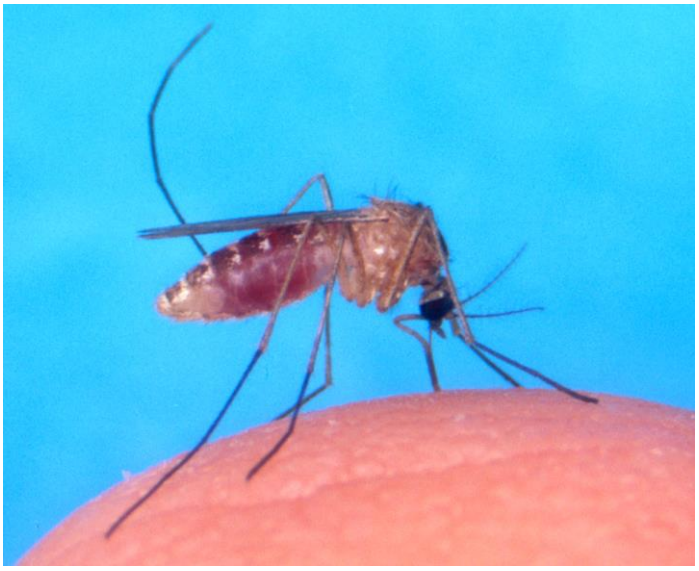

Figure 1-3. *Culex pipiens quinquefasciatus*.
Image provided courtesy of Dr. Stephen Higgs.

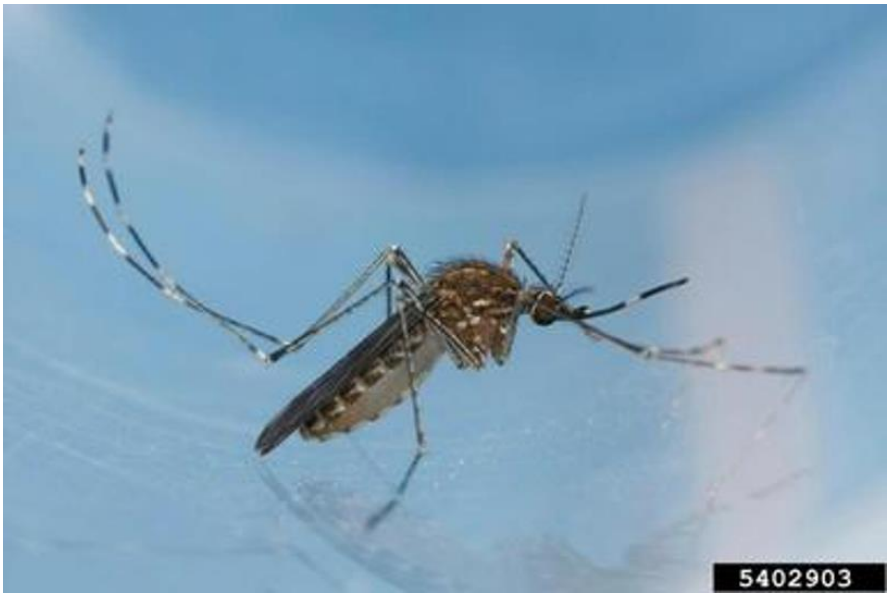

Figure 1-4. *Culex tarsalis*.
Image from Joseph Berger, Bugwood.org; Published under the Creative Commons Attribution License**.**

As discussed above, mosquitoes from these genera are able to transmit a number of viruses primarily in the *Alphavirus* and *Flavivirus* genera but are still considered important vectors for the transmission of viruses in the *Orthobunyavirus* and *Phlebovirus* genera. Since mosquitoes are found worldwide, except Antarctica, mosquito-borne viruses exist in most parts of the world. These viruses are maintained and amplified in cycles involving transmission of the virus from mosquitoes to small mammals, amphibians, birds, equines, ruminants, non-human primates, and/or humans. The section below focuses on the transmission of arboviruses from competent mosquitoes to susceptible hosts.

Arthropod-borne transmission

Arthropod-borne viruses are viruses which are maintained in nature principally, through biological transmission between susceptible vertebrate hosts by blood feeding arthropod vectors (World Health Organization, 1967). There are several overlapping transmission cycles that have been described for arthropod-borne viruses. The first one is the sylvatic/endemic cycles which refers to the natural maintenance transmission cycle of a pathogen but can result in incidental human and agricultural animal infections. Some arboviruses can be further amplified in a rural/epizootic cycle which is associated with the emergence of endemic arboviruses. Lastly, the urban/epidemic cycle is supported by peridomestic mosquito-borne transmission between viremic humans. **Figure 5-1** is an example of how an arbovirus can be involved in some of these different transmission cycles.

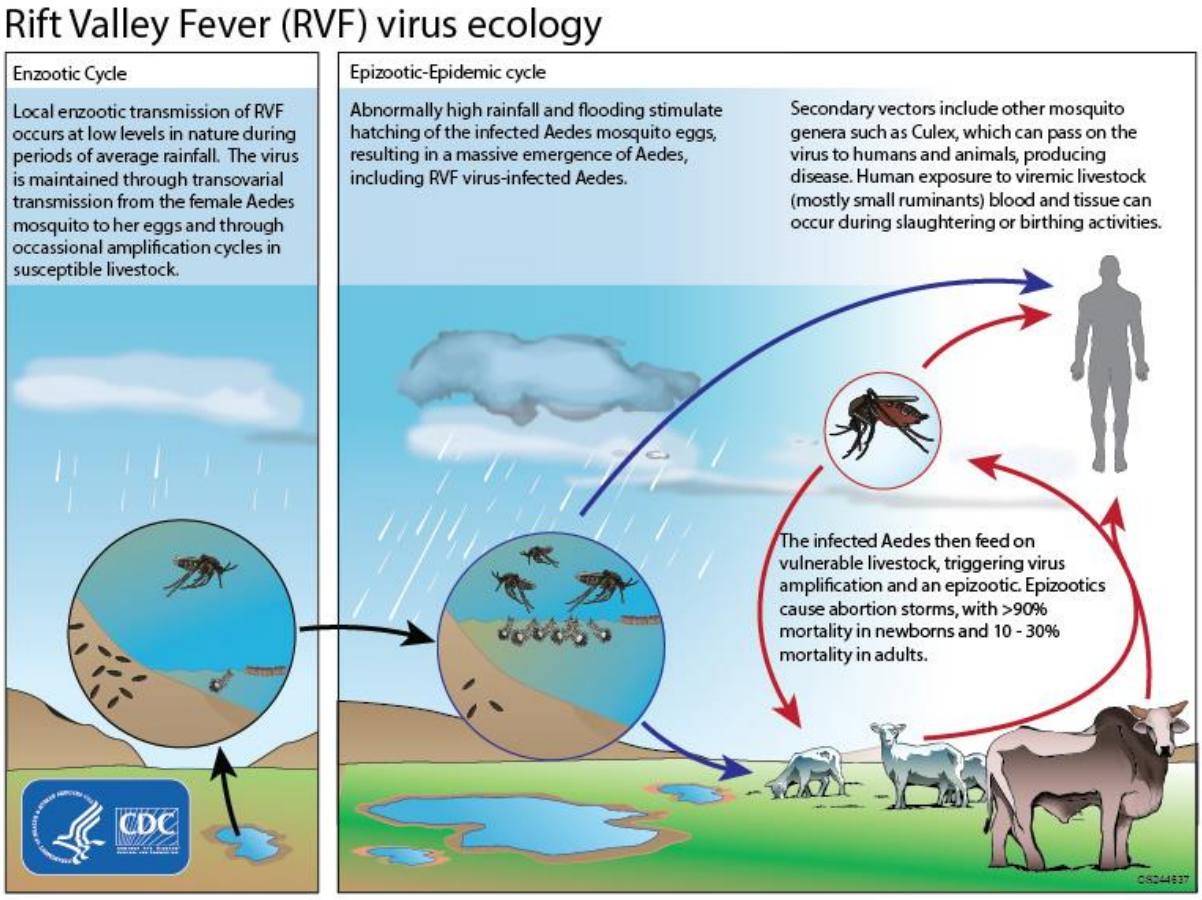

Figure 1-5. Rift Valley fever virus ecology

Image from (Centers for Disease Control and Prevention, 2020c); published under the public domain.

Mosquitoes are involved in these overlapping transmission cycles due to the female arthropods initially searching for blood meals as a supplement for growing their eggs (Stone et al., 2011). Infection of hematophagous arthropods is dose-dependent following ingestion of an infectious blood meal, therefore only vertebrate hosts with a sufficient viral titer can contribute to the transmission of that virus (Kenney & Brault, 2014). Nonviremic transmission has previously been described as a phenomenon by which arthropod vectors become infected with a

pathogen before its propagation in the host and its appearance in the circulatory system (Higgs et al., 2005).

Once feeding occurs, multiple steps must take place in order for the mosquito to transmit the virus to a susceptible host. The blood meal first enters into the midgut where the virus must infect the epithelial cells to replicate (Romoser et al., 2004). To develop a disseminated infection, the virus then infects the secondary tissues (head, wings, and legs) and organs, and most importantly, the salivary glands. Typically, after replication and dissemination, the arthropod will feed on a vertebrate host and inject saliva and infectious viruses (Kuno & Chang, 2005). If the vertebrate host becomes infected, the period from infection to disease is called the intrinsic incubation period (Higgs et al., 2005).

Transmission of viruses from an arthropod vector to a suitable host can also be non-biological (direct or mechanical). Direct transmission can occur when insectivorous mammals such as bats orally consume an infected mosquito, which has been documented to occur with RVFV (Kuno & Chang, 2005). Another nonbiological transmission route is mechanical transmission, which occurs when a vector transfers a virus from its contaminated mouthparts while switching hosts during feeding, which has also been documented to occur with RVFV (Kuno & Chang, 2005).

Some arboviruses, especially those in the genus *Orthobunyavirus* and *Phlebovirus*, have been transmitted transovarially (Kuno & Chang, 2005). Transovarial transmission refers to transmission of an infectious virus from the arthropod vector to its offspring (Lequime et al., 2016). This type of transmission was first described and best characterized for LaCrosse virus infection of *Ae. triseriatus* but has now been demonstrated for a number of arboviruses (Watts & Eldridge, 1975; Watts et al., 1973; Watts et al., 1975). Horizontal transmission does not only

occur from arthropod to vertebrate hosts but can also occur between a male and female vector through venereal transmission (Nadim et al., 2020). In addition, horizontal transmission can occur between two mosquitoes during co-feeding on a non-viremic host (Higgs et al., 2005).

Vector competence and forced salivation

The transmission of an arbovirus relies on competent vector species and susceptible hosts. For an arthropod vector to be competent for transmission it should have the intrinsic ability to biologically transmit a pathogen (Higgs et al., 2005). This can be evaluated through vector competence studies, where the infection, dissemination, and transmission rates are determined. To ascertain the incidence of dissemination, dissection of mosquitoes is performed to separate the abdomen (contains the midgut) from the head, wings, and legs (secondary tissues) (**Figure 1-6**). The midgut is examined for primary infection whereas the secondary tissues are examined for dissemination and amplification of the virus.

Another way to determine dissemination of the virus and to also look at the transmission rate is by examining the mosquito's saliva. Saliva is obtained from mosquitoes through forced salivation, as previously described (Huang et al., 2016; Vanlandingham et al., 2004). Part of this dissertation is focused on the virus-vector interactions between an emerging bunyavirus and medically important mosquito species by utilizing vector competence studies. Investigating the vector competence of medically important mosquito species in North America will determine what species may be involved in the different overlapping transmission cycles. More specifically, vector competence studies can help assess which species are responsible for the enzootic spillover of CVV. Vector competence studies can also assess the likelihood of non-endemic viruses establishing their transmission cycle in new territories. Therefore, it is critical

that North American vectors are assessed for their potential to transmit emerging bunyaviruses to improve current or create new mitigation strategies.

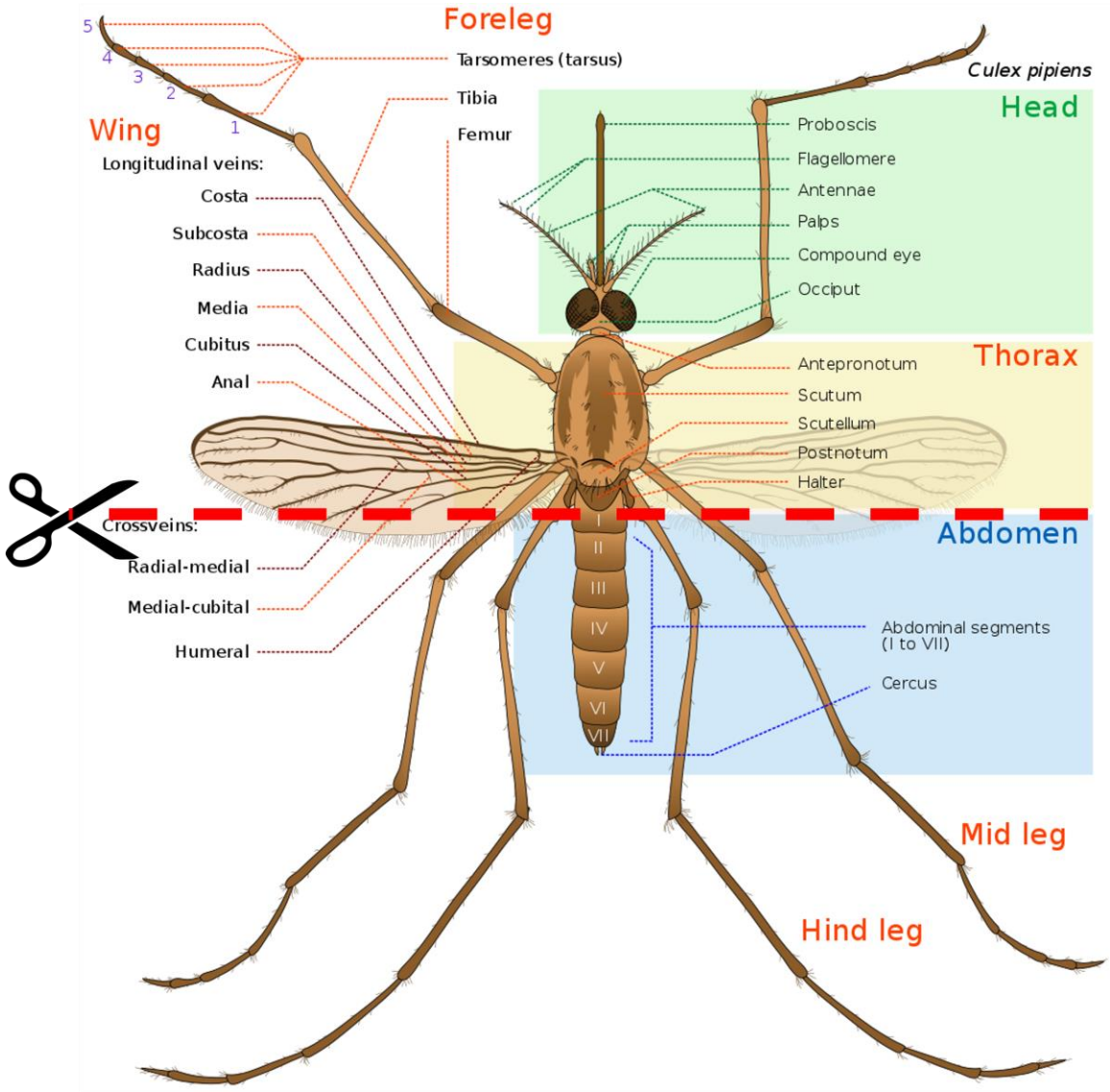

Figure 1-6. Mosquito dissection.

Dissected mosquitoes were separated at the red dashed line. This allowed for the abdomen containing the midgut to be separated from the head, wings, and legs. Image modified from Mariana Ruiz Villarreal and published under the Creative Commons Attribution License.

Cache Valley virus

Introduction

Cache Valley virus is a zoonotic mosquito-borne virus in the genus *Orthobunyavirus*, order *Bunyavirales*, and family *Peribunyaviridae*. Cache Valley virus was first isolated from *Culiseta* (*Cs.*) *inornata* mosquitoes in Cache Valley, Utah in 1956 during routine testing for Western and St. Louis encephalitis virus from wild-caught mosquitoes (Holden & Hess, 1959). Cache Valley virus has since been isolated from over 30 species of mosquitoes, such as *Aedes*, *Culex*, and *Anopheles* species, and is now regarded as the most widely distributed member within the Bunyamwera serogroup in the New World (Campbell et al., 1992).

Cache Valley virus is endemic in North America, but circulates in mosquitoes and vertebrate hosts throughout Central America and parts of South America with several known regional subtypes, including Fort Sherman virus, Tlacotalpan virus and Cholul virus (Skinner et al., 2022). Cache Valley virus was first recognized in sheep causing embryonic deaths and stillbirths (Edwards, 1994). Cache Valley virus has also been isolated from blood samples of horses and white-tailed deer (McLean et al., 1987; Neitzel & Grimstad, 1991). The impact on the domestic and wild animal population was unknown and is still not well-defined.

Whilst CVV is known as an important agricultural pathogen, it began to be recognized as a human pathogen in the late 1990s, causing rare but severe disease in humans (Nguyen et al., 2013; Sexton et al., 1997). Not only CVV, but its related subtypes and other related viruses such as, Maguari virus and Fort Sherman virus can cause human disease (Groseth et al., 2017). However, there is a major gap in knowledge in how this zoonotic spillover takes place as the epizootic vectors and mammalian reservoirs for CVV transmission have rarely been investigated.

Given the high seroprevalence rates in regions of North America (as high as 96.7% in the eastern U.S., 53.3% in the central U.S., and 58.9% in the Western U.S.), and the continuing geographic expansion of competent mosquito vectors, the risk of epidemic and epizootic emergence of CVV is high, and interventions are needed for this important pathogen (Meyers et al., 2015).

Transmission cycle

When creating a disease mitigation strategy, understanding how a virus is maintained and transmitted is critical. The CVV transmission cycle is maintained in an animal-mosquito cycle, as shown in **Figure 1-7**, however our understanding of CVV ecology and transmission remains limited. Data from serological surveys have indicated that there are multiple amplification hosts for CVV, with some having agricultural importance (Blackmore & Grimstad, 1998; Campbell et al., 1989; Eldridge et al., 1987; Neitzel & Grimstad, 1991). An amplification host is an organism that can develop high and sometimes prolonged viremia capable of infecting arthropod vectors, allowing the virus to enter into urban outbreak cycles (Weaver, 2005). It is assumed that the transmission of CVV is primarily maintained amongst white-tailed deer (*Odocoileus virginianus*) in nature (Blackmore & Grimstad, 1998; Friesen & Johnson, 2013; Mehus & Vaughan, 2013). With over 4,000 white-tailed deer operations in America and the large population of wild deer, transmission of CVV is ubiquitous, which could be part of the cause for epizootic transmission to humans (Brooks et al., 2015). Other suggested amplifying hosts of CVV are livestock, especially pregnant sheep, goats, and cattle (Blackmore & Grimstad, 1998; Blitvich, Lorono-Pino, et al., 2012; Calisher et al., 1986; Campbell et al., 2006). Humans are considered dead-end hosts, meaning that they cannot produce sufficient viremia capable of direct transmission.

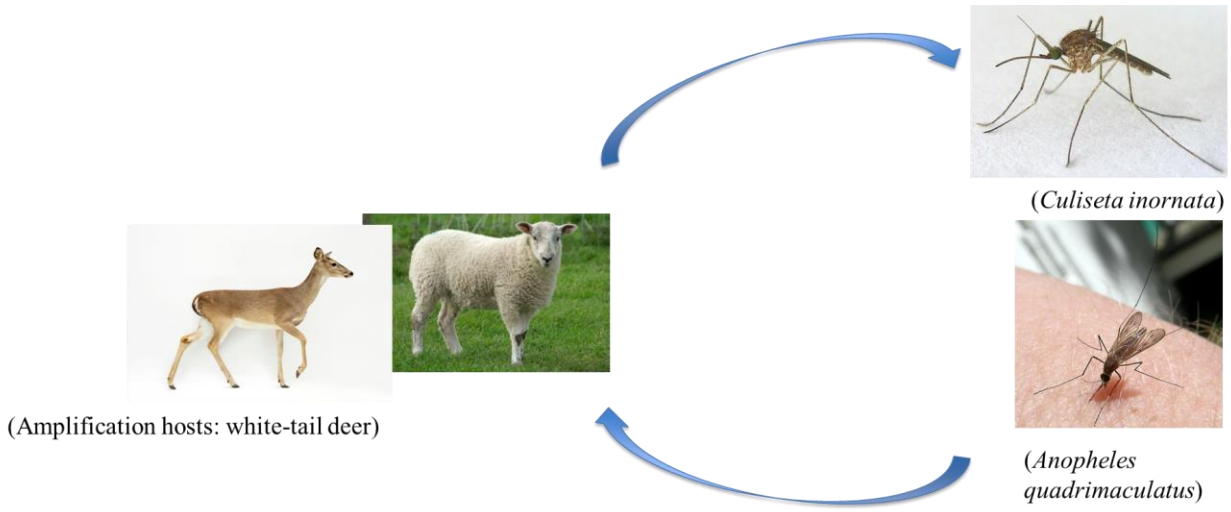

Figure 1-7. The Cache Valley virus transmission cycle.
Maintained in animal – mosquito cycles.

CVV is an arbovirus making it primarily transmitted to vertebrate hosts and humans from the bites of infected mosquitoes (Lopez et al., 2021). The principal vectors for CVV are unknown, though CVV has been isolated from many species of field-caught mosquitoes, with 146 wild-caught mosquito species investigated (Waddell et al., 2019). Most species that transmit CVV are known to be multivoltine and mammalophilic, however, some species that have been studied for vector competence under laboratory settings prefer humans. These data suggest how mosquitoes play a role in the transmission of CVV in the domestic and peridomestic settings of urban and suburban areas. Since intravenous inoculation of CVV in sheep only produces low-grade viremia with no *in utero* infection, vector factors may play a role in the development of clinical disease (Edwards, 1994). In fact, without the saliva from some mosquito species, mice are not susceptible to CVV infection under laboratory conditions (Edwards et al., 1998).

Similar to other orthobunyaviruses, vertical transmission has been demonstrated for CVV, likely as an overwintering mechanism (Hayles & Lversen, 1980; F. Yang et al., 2018).

While the principal vector for CVV is still being investigated, field isolations and vector competence studies have demonstrated that *Anopheles* (*An.*) *punctipennis* and *An. quadrimaculatus* may play an important role in the natural transmission cycle (Andreadis et al., 2014; Blackmore et al., 1998).. Under laboratory condition, confirmed competence has been reported for several species from the *Aedes* (*Ae.*), *Culex* (*Cx.*), and *Culiseta* (*Cs.*) genera, as shown in **Table 1-1** (Hayles & Lversen, 1980; Waddell et al., 2019; Y. Yang et al., 2018). Since the relative importance of different competent vector species for CVV is still unknown, identifying competent vectors that can support the epizootic transmission of CVV will improve our understanding of how this pathogen causes human disease.

Table 1-1. Vector competence experiments with Cache Valley virus and Cache Valley-like virus.

Species	Infection (%)	Dissemination (%)	Transmission (%)	References
Aedes albopictus	1.2 (44 total)	N/A	N/A	(Miller, 1997)
Aedes communis group	0 (0/35)	N/A	0 (0/34)	(E. K. Saliba et al., 1973; Elias K. Saliba et al., 1973)
Aedes japonicus japonicus	41 (74)	38 (74)	28 (74)	(F. Yang et al., 2018)
Aedes punctipennis	90 (9/10)	N/A	20 (2/10)	(E. K. Saliba et al., 1973)
	85 (17/20)	N/A	30 (6/20)	
	31 (5/16)	N/A	0 (0/16)	
Aedes scapularis	N/A	N/A	90.5 (19/21) (transmission to mice)	(Aitken & Spence, 1963)
Aedes serratus	N/A	N/A	10 (1/10) (transmission to mice)	(Aitken & Spence, 1963)
Aedes sollicitans (wild-caught)	80 (20/25)	N/A	50 (4/8)	(Yuill & Thompson, 1970)
	70 (19/25)	N/A	60 (3/5)	
	59 (16/27)	N/A	0 (0/5)	
Aedes sticticus	40 (8/20)	N/A	0 (0/20)	(E. K. Saliba et al., 1973)
	65 (13/20)	N/A	0 (0/20)	
Aedes taeniorhynchus	85 (11/13)	N/A	27.3 (3/11)	(Yuill & Thompson, 1970)
	100 (38/38)	N/A	50 (4/8)	
	67 (14/21)	N/A	11.1 (2/18)	

Number alone in parentheses is the total sample size

Table 1-2. Vector competence experiments with Cache Valley virus and Cache Valley-like virus.

Species	Infection (%)	Dissemination (%)	Transmission (%)	References
Aedes taeniorhynchus (wild-caught)	100 (15/15)	N/A	100 (3/3)	(Yuill & Thompson, 1970)
	100 (10/10)	N/A	50 (1/2)	
	50 (5/10)	N/A	66.7 (2/3)	
Aedes triseriatus	0 (0/28)	N/A	0 (0/28)	(E. K. Saliba et al., 1973)
	0 (0/32)	N/A	0 (0/32)	
Aedes vexans	43 (10/23)	N/A	0 (0/24)	(E. K. Saliba et al., 1973)
Anopheles quadrimaculatus	81 (53)	81 (53)	18 (22)	(Blackmore et al., 1998)
	100 (42)	100 (42)	19 (32)	
	100 (50)	100 (50)	20 (50)	
	100 (30)	100 (30)	33 (30)	
	100 (30/30)	N/A	0 (0/27)	(E. K. Saliba et al., 1973)
	44 (14/32)	N/A	0 (0/32)	
	93 (25/27)	N/A	30 (8/27)	
	92 (11/12)	N/A	08 (1/12)	
	100 (23/23)	N/A	47 (8/17)	
	100 (27/27)	N/A	37 (10/27)	
	100 (9/9)	N/A	0 (0/9)	
	100 (10/10)	N/A	20 (2/10)	
	98 (44/45)	N/A	04 (2/45)	
	0 (0/16)	N/A	0 (0/16)	
	37 (10/27)	N/A	0 (0/25)	
	43 (12/28)	N/A	0 (0/27)	
Coquillettidia perturbans	90 (40)	75 (40)	8 (26)	(Blackmore et al., 1998)
	98 (45)	91 (45)	45 (38)	
	20 (40)	10 (40)	0 (4)	
	44 (36)	36 (36)	25 (12)	
	95 (39)	93 (39)	39 (28)	
	90 (29)	90 (29)	67 (24)	
Culex nigripalpus	N/A	N/A	100 (4/4) (transmission to mice)	(Aitken & Spence, 1963)
Culex pipiens quinquefasciatus	N/A	N/A	79 (15/19) (transmission to mice)	(Aitken & Spence, 1963)
Culicoides sonorensis	93 (28/30)	0	N/A	(Reeves & Miller, 2013)

Number alone in parentheses is the total sample size

Clinical Disease

Clinical outcomes in animals

CVV is an important agricultural concern that causes significant agroeconomic losses, even though no research has truly investigated the economic burden from the disease (Lopez et al., 2021). Cache Valley virus is clinically most significant in the sheep industry, due to severe embryonic lethality associated with infection (Uehlinger et al., 2018). Currently, small ruminants, predominantly sheep and goats, are the only animals in which clinical disease for CVV has been extensively studied. Under laboratory conditions, clinical signs in CVV-infected ewes and goats are typically subclinical but appetite loss, elevation of body temperature, and disorientation has been reported (McConnell et al., 1987). Cache Valley virus infections usually result in dystocia, congenital defects, and embryonic death in sheep and to a lesser extent goats during pregnancy (de la Concha-Bermejillo, 2003).

The deformities that occur usually depend on the time of the infection during pregnancy though. Fetuses that were inoculated in the earlier phases of gestation, days 27 – 35, had the greatest mortality, and those infected between days 36 and 45 more commonly had malformations (de la Concha-Bermejillo, 2003). Fetuses older than 49 days that were inoculated resulted in no abnormalities (de la Concha-Bermejillo, 2003). Most abnormalities or abortions caused by CVV occur when the ewe is infected with CVV 30 – 45 days after breeding. During another experiment involving CVV-infected ewes, fetuses were collected, and gross findings were observed and compared between control and infected ovine fetuses (**Figure 1-8**). The control fetuses floated freely within the amniotic sac whereas CVV-infected fetuses resulted in the fetal amnion becoming adherent to the body of the fetus, scoliosis of the cervical and thoracic vertebrata, and severe arthrogryposis (Rodrigues Hoffmann et al., 2012).

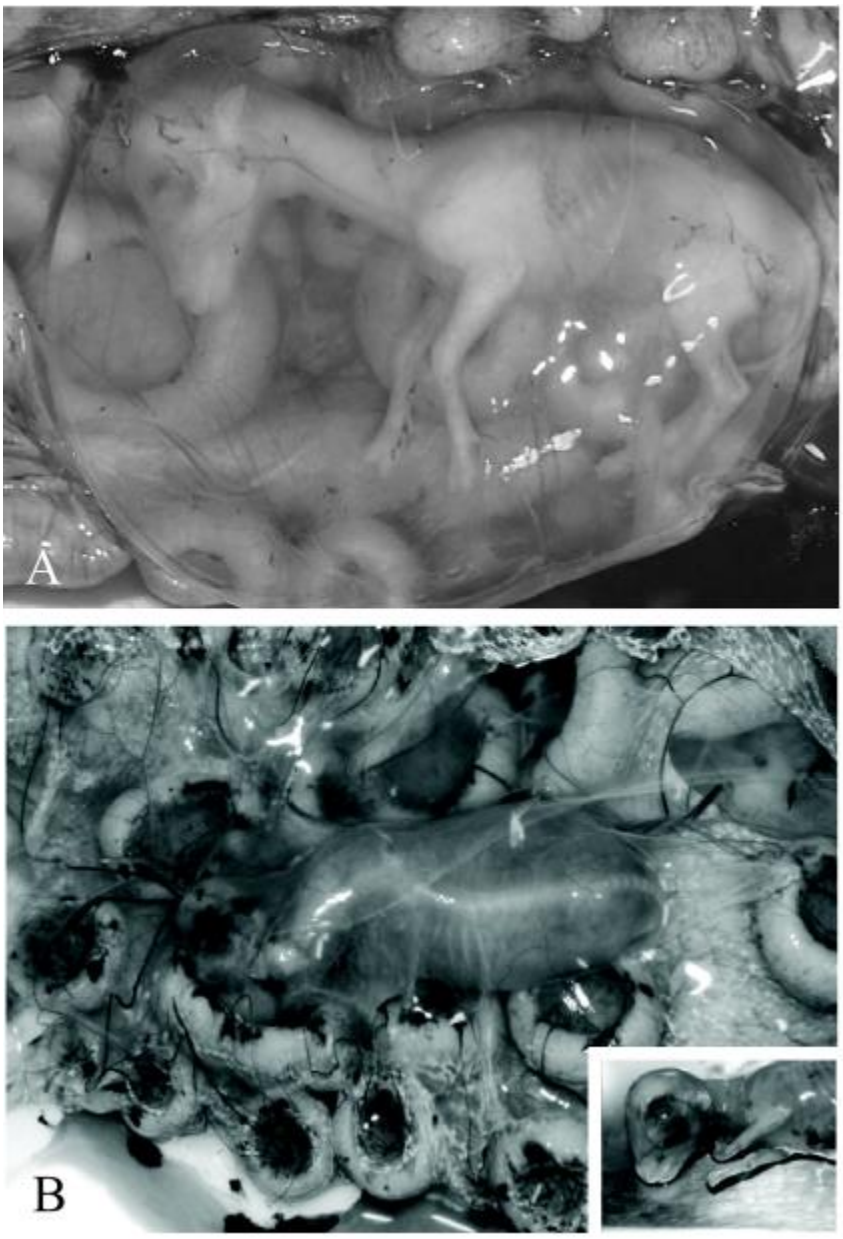

Figure 1-8. Gross findings in control and infected ovine fetuses.

(A) Control fetus at 56 pbd. The fetus floats freely within the amniotic sac. (B) Ovine fetus infected with CVV, 21 dpi (56 pbd). Severe oligohydramnios results in the fetal amnion becoming in close contact with the body of the fetus. The fetus has scoliosis of the cervical and thoracic vertebrae. (Inset) Severe arthrogryposis results in hypercontraction of the limbs. Image used with permission from (Rodrigues Hoffmann et al., 2012), published by the American Society for Microbiology in the *Journal of Virology*.

Outbreaks of CVV in sheep have been reported during the months of December and February, which is lambing season (Crandell et al., 1989; Edwards et al., 1988). These outbreaks took place in Texas, USA, and Ontario, Canada, and resulted in stillbirths and fetal deformities (Chung, Livingston, Edwards, Gauer, et al., 1990; Edwards et al., 1989; Shapiro et al., 2012). Amongst cattle and horses, clinical signs are rare and CVV prevalence remains unknown. Understanding the biology of CVV and improving awareness among veterinarians could provide a better understanding of how CVV infections affect livestock populations.

Clinical outcomes in humans

Shortly after Cache Valley virus was recognized as a human pathogen causing rare but severe disease in humans, where a correlation between maternal antibodies against CVV and incidence of microcephaly and macrocephaly in infants was observed (Calisher & Sever, 1995). However, the potential teratogenic effects caused by CVV in humans has not been well defined since. Currently, six human cases of CVV have been reported, all occurring in the United States. This low number is most likely because diagnosis for CVV in humans is rarely made, however, seroprevalence has been reported to be as high as 50% in North and South America (Blitvich, Saiyasombat, et al., 2012; Downs et al., 1961; Work, 1964). CVV-associated symptoms are initially non-specific, progressing to more severe clinical signs. A brief description of how the infection progressed in these human cases is described below.

The first case was reported in 1995 for a 28-year-old, previously healthy man who had fever, chills, headache, and myalgias (Sexton et al., 1997). Symptoms became extremely severe as he experienced confusion, respiratory failure, and a leg amputation (Sexton et al., 1997). Three months post infection, the patient was still unable to walk or speak. The patient then died around 8 months post infection. The second human case occurred in a 41-year-old Wisconsin

man who was previously healthy as well. He experienced acute aseptic meningitis but improved three days later with full recovery reported (Campbell et al., 2006). The third case involved a 63-year-old woman in New York who presented fever, headaches, neck stiffness, and photophobia (Nguyen et al., 2013). Symptoms began to become more severe but gradually improved and was discharged from the hospital four days after admission (Nguyen et al., 2013). Although partially recovered, the patient continued to have difficulty finding words, experienced severe headaches, and continued to require medical care (Nguyen et al., 2013). The fourth case reported occurred in Missouri but was only described in a surveillance report with limited case information (Centers for Disease Control and Prevention, 2017). Another case occurred in a 58-year-old-man, also from New York, who was experiencing memory loss, fatigue, and weight loss (Y. Yang et al., 2018). This patient had a history of travel and was immune compromised. He developed bulky lymphadenopathy, had to receive treatments every six months, and died almost two years later (Y. Yang et al., 2018). The last known case was reported in a man from Australia who had recently traveled to the United States had chronic meningoencephalitis and neurological deficits. His Montreal Cognitive Assessment score was 7/30 with severe impairment in visuospatial and executive function, attention, abstraction, and delayed recall (Nasreddine et al., 2005). He was placed in hospice care and eventually passed away, 42 months after his initial presentation (Wilson et al., 2017).

Although the number of human cases is small, the symptoms are diverse and can be devastating with no specific treatments available. According to the seroprevalence rates reported for CVV, exposure to the pathogen seems to occur more frequently than the clinical disease. However, there is still insufficient evidence to know how under-diagnosed CVV is and to know how diverse the clinical signs may be. Most of the patients in these cases were likely exposed to

CVV by infected mosquitoes in nature, which is potentially what caused this enzootic spillover. Advancement in the understanding of the biology of CVV as well as CVV vectors could improve human health and well-being. Therefore, part of this dissertation work evaluates vector competence of medically important mosquito species in North America. Additionally, a candidate live-attenuate vaccine for CVV was investigated to further the advancement of future prevention strategies.

Rift Valley Fever virus

Introduction

Rift Valley fever virus is a zoonotic mosquito-borne virus in the genus *Phlebovirus*, order *Bunyavirales*, and family *Phenuiviridae*. Rift Valley fever virus was first isolated in 1931 in the Rift Valley in Kenya during an epidemic among sheep (Daubney & Hudson, 1932; Horne & Vanlandingham, 2014; Kwasnik et al., 2021). However, case reports of an illness in sheep consistent with RVF disease was described in 1910 (Bird et al., 2007). Rift Valley fever virus was first identified in 1931 during an investigation of an epidemic among sheep on a farm in the Rift Valley of Kenya (Daubney et al., 1931). Thousands of sheep died during the 1931 epidemic, with the majority of the fatalities occurring three to seven days after birth (Daubney et al., 1931).

Rift Valley fever virus has since been endemic in South Africa, Zimbabwe, Senegal, Madagascar, Egypt, and the Arabian Peninsula, as shown in **Figure 1-9**, with the ability to cause severe animal and human disease (Centers for Disease Control and Prevention, 2020b; Coetzer, 1982; Dar et al., 2013; Guillaud et al., 1988; Meegan, 1979; Swanepoel, 1981). The occurrence of outbreaks has led to significant animal and human loss as well as socioeconomic impacts. For

example, the epizootic of 1931 in South Africa led to over 100,000 sheep deaths and a half a million livestock abortions (Daubney et al., 1931). Most RVF outbreaks are linked to heavy rainfall and local flooding (LaBeaud et al., 2008). This is likely because infected mosquito eggs can hatch on the edge of damboes during rainy season, infecting nearby humans and animals (Horne & Vanlandingham, 2014). An outbreak in the Horn of Africa that was closely related to flooding resulted in an estimated 500 human deaths and thousands of livestock deaths in 1997 – 1998 (Woods et al., 2002). After flooding due to heavy rainfall in 2000, there was also an outbreak of RVFV in Saudi Arabia and Yemen, resulting in over 200,000 human infections and thousands of livestock deaths (Abdo-Salem et al., 2006; Balkhy & Memish, 2003).

These outbreaks have been considered to be related to climate conditions, periodic flooding, and the emergence of mosquito vectors with the ability to infect susceptible ruminant hosts (Nanyingi et al., 2015). Although there was a formalin-inactivated vaccine once available for use in humans in the 1970s, there are currently no licensed vaccines for the prevention or control of RVFV in humans (Bird et al., 2009). There are several licensed veterinary vaccines available, but they all have limitations in both efficacy and safety, which will be described in a later section (Bird et al., 2009; Ikegami & Makino, 2009; Indran & Ikegami, 2012; Kortekaas, 2014; Pepin et al., 2010).

Rift Valley Fever Distribution Map

Countries reporting endemic disease and substantial outbreaks of RVF

Countries reporting few cases, periodic isolation of virus, or serologic evidence of RVF infection

RVF status unknown

0 250 500 1,000 Miles

0 375 750 1,500 Km

Figure 1-9. Geographic distribution of RVFV.

Image used from (Centers for Disease Control and Prevention, 2020b); Published in the public domain.

According to the Centers for Disease Control and Prevention (CDC) and the United States Department of Agriculture (USDA), RVFV is considered a Category A pathogen, meaning it has the potential to pose a severe threat to both human and animal health, and is recognized as an important potential bioterrorism agent (Centers for Disease Control and Prevention, 2021; Mandell & Flick, 2011). The possible introduction of RVFV into new geographic areas is an important public and veterinary health concern. Although RVFV is mostly found in Africa, the presence of competent arthropod vectors and susceptible amplifying hosts in other geographic regions could potentially lead to an introduction and establishment into new areas. These reasons plus the prospective use for bioterrorism presents the urgent need for developing a safe and efficacious vaccine for both veterinary and human use.

Transmission cycle

The RVFV transmission cycle is multiplex, involving mosquitoes, livestock, humans, and the environment (**Figure 1-10**). There are two cycles that can be involved in the maintenance and transmission of RVFV: the enzootic cycle and the epidemic-epizootic cycle (Fawzy & Helmy, 2019). Enzootic is a disease or maintenance transmission cycle occurring continuously among non-human animals in a particular region (Weaver & Barrett, 2004). The RVFV enzootic cycle occurs in the enzootic area of Africa during normal rainfall where RVFV is present in a silent infection cycle and emerges after rainfall to start the disease epizootics (Fawzy & Helmy, 2019). The RVFV enzootic cycle typically involves *Aedes* species mosquitoes with the ability to transmit RVFV vertically to their offspring or through occasional amplification in wildlife (Bird et al., 2009; Fawzy & Helmy, 2019; Hartman, 2017; Linthicum et al., 2016). Epizootic is when there is higher than average amplification, or occurrence, of a disease or pathogen in non-human

animals. The epidemic-epizootic cycle occurs during excessive rainfall and flooding of dams. Usually during this cycle, *Culex* species mosquitoes transmit RVFV transovarially (Fawzy & Helmy, 2019). After heavy rainfall, the infected mosquito eggs hatch and the infected adult mosquitoes then feed on and infect wild animals. Since mosquito breeding depends on rainfall, outbreaks are usually linked to the environment and can be affected by climate change (Nanyingi et al., 2015).

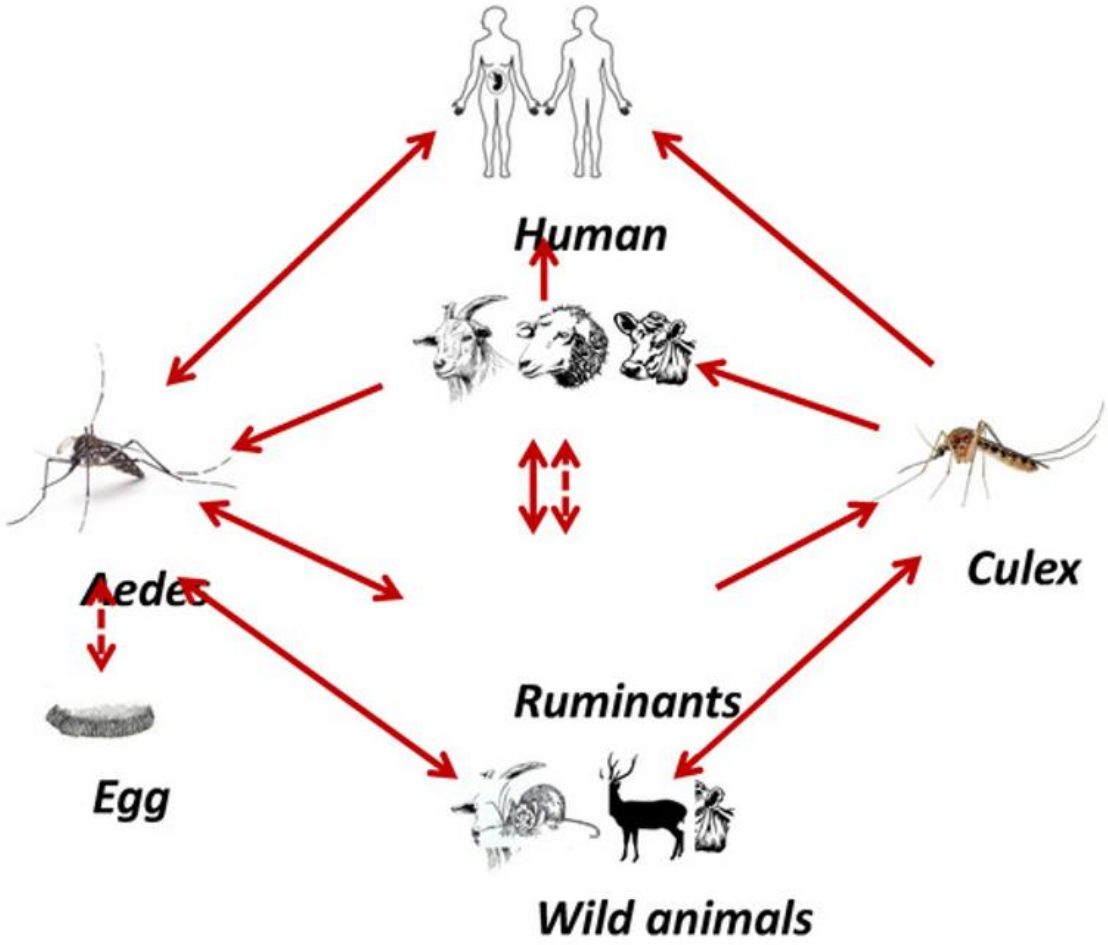

Figure 1-10. The life cycle of the RVF virus in Egypt and the role of the mosquito vectors.

There are two transmission cycles for RVFV in nature: (1) an enzootic cycle that can occur during the normal rainfall and involves the *Aedes* mosquitoes, which transmit the virus vertically to their offspring, and (2) an epidemic-epizootic cycle that occurs during abnormally heavy rainfall and flooding of dams or during the warm season. The virus is transmitted transovarially and the *Culex* mosquitoes distribute the virus and induce the emergence of outbreaks. The transmission of the virus to humans occurs by direct contact with infected animals. The continuous line represents the direct transmission, while the dashed line represents the vertical transmission. (Image used from (Fawzy & Helmy, 2019); Published under the Creative Commons Attribution License.)

Flooding caused by heavy rainfall creates an increased interaction between mosquitoes and domesticated livestock (the amplifying host) (Hartman, 2017). Once livestock are infected, non-infected mosquitoes can feed on the viremic host and potentially transmit the virus to other

susceptible hosts. Direct transmission among animals has also been suggested but has not been experimentally proven (Chevalier et al., 2011). Direct transmission increases the risk of outbreaks, especially in densely populated livestock areas. The most effective way to reduce this risk in livestock is through vaccination against RVFV.

Previous studies have identified multiple mosquito species as competent vectors for RVFV, with *Aedes* as the primary vector and *Anopheles*, *Culex*, and *Mansonia* as important secondary vectors that can contribute to amplification of epidemics and epizootics (Clements, 2011; Linthicum et al., 2016). The virus can also infect humans after contact with infected animals, through inhalation of aerosols produced during animal slaughter, and through ingestion of infected uncooked animal products (Javelle et al., 2020). Although the virus has not shown human-to-human transmission, there have been a few cases reported of vertical transmission (Adam & Karsany, 2008; Arishi et al., 2006; Niklasson et al., 1987). However, there is still a need for studies toward the understanding of RVFV ecology for better prevention and mitigation strategies.

Clinical disease

To determine RVFV host range, early experiments showed that non-ruminants such as birds, horses, rabbits, and pigs were resistant to Rift Valley fever (RVF), but that mice, rats, and hamsters were highly susceptible (Findlay, 1932; Ikegami & Makino, 2011; Scott, 1963). Since then, mice and rats have been used as the main animal model to conduct RVFV pathogenicity and vaccine efficacy studies, while RVF in livestock has mostly been characterized anecdotally during outbreaks with some laboratory-based experimental studies.

Clinical outcomes in mice

Mice are the most widely used animal model to study RVF pathogenesis and vaccines because they are highly susceptible, cost-effective, and mirror the disease seen in ruminants (Findlay, 1932; Ikegami & Makino, 2011). The clinical symptoms occur around 2-3 days post infection and are characterized by ruffled fur, hunched posture, and lethargy (Smith et al., 2010). Mice infected with wild-type RVFV ZH548 or ZH501 strains usually die in 3-5 days but can die even faster when infected with other wild-type strains (Bouloy et al., 2001; Smith et al., 2010; Vialat et al., 2000).

Clinical outcomes in livestock

RVFV was first reported in 1931 with abortions in sheep followed by unusually high mortality in newborn lambs (Daubney et al., 1931). Ruminants are now the most applicable model for studying RVF pathogenesis and vaccines since they are the natural host for RVFV. However, the lack of large animal biocontainment facilities can limit their usefulness. Despite this limitation, RVF infection in sheep can be studied in both laboratory settings and in nature.

Sheep are the most susceptible to RVF infections where the clinical symptoms during outbreaks are usually characterized by “abortion storms” in pregnant ewes (Daubney et al., 1931). Abortion storms are when the transmission of RVFV is rapid and includes widespread abortions in herds of pregnant sheep causing up to 100% neonatal mortality (Bird, Githinji, et al., 2008; Coetzer, 1982; Easterday, 1965). Clinical symptoms in adult sheep can include high fever, nasal and ocular secretions, abdominal colic, and abortions; and experimentally, adult sheep mortality after RVF infection is between 20 and 30% (Easterday, 1965; Ikegami & Makino, 2011). Although RVF in neonatal lambs is nearly uniformly fatal, the susceptibility in adult

sheep varies and can depend on the RVFV strain and breed of sheep (Busquets et al., 2010; Daubney et al., 1931; Easterday, 1965; Olaleye et al., 1996; Tomori, 1979).

In contrast, the disease in goats is more variable and the viremia and symptoms are inconsistent after infection (Kwasnik et al., 2021). Cattle are also less susceptible to RVF disease than sheep and goats (Kwasnik et al., 2021). Rift Valley fever infection is usually asymptomatic in adult cattle but, acute disease is possible with a mortality rate of up to 5% (Ikegami & Makino, 2011; Wilson et al., 2016).

Clinical outcomes in humans

RVF infections in humans can range from asymptomatic to severe disease. The incubation period is typically four to six days (Ikegami & Makino, 2011). Uncomplicated flu-like signs occur in 50 to 95% of infected cases, with symptoms such as fever, headache, sweating, weakness, and joint or muscle aches (Laughlin et al., 1979). However, a small percentage of symptomatic cases can present complications and more severe disease, leading to death (Ikegami & Makino, 2011). This can vary though, for example, the major outbreak in Egypt in 1977 reported less than 5% of symptomatic cases, whereas the outbreak in South Africa in 1975 and in Tanzania is 2007 presented with encephalopathy in 71% and 89% of cases respectively (McIntosh et al., 1980; Mohamed et al., 2010).

Severe pathological forms that can occur include hemorrhagic fever, thrombosis, and neurological disease (Javelle et al., 2020; Mohamed et al., 2010). Hemorrhagic fever typically starts suddenly but can appear two to four days after the onset of the illness, presenting symptoms such as vomiting blood, bleeding in the skin, or bleeding from the nose, gums, or eyes (Ikegami & Makino, 2011; Javelle et al., 2020; Yassin, 1978). Patients who recover from severe RVF illnesses are recommended to schedule regular follow-ups for at least one month after the

onset of symptoms due to the possibility of delayed neurological and/or ocular complications (Javelle et al., 2020). It has been reported that viremic loads correlate with severe RVFV, but actual determinants are poorly defined (Njenga et al., 2009). Preventing and controlling outbreaks through a vaccination program is the best way to mitigate the disease.

CVV and RVFV Vaccines

Despite the growing importance of these two pathogens there are currently no licensed vaccines or treatments for animal or human use available for the prevention or control of CVV and although there are veterinary vaccines for RVFV, they have safety and distribution limitations. Additionally, the knowledge to develop a CVV and RVFV vaccine is an important step in understanding how to control other bunyaviruses. The rest of this section will describe the vaccines available and previously used for RVFV and their limitations.

There are currently no licensed vaccines commercially available for use in humans. An inactivated vaccine has been developed but only used to experimentally protect veterinary and laboratory personnel at high risk of RVF exposure (World Health Organization, 2022). While there are a variety of veterinary vaccines for RVFV, there are no fully licensed RVFV vaccines approved for veterinary use outside endemic areas (Bird et al., 2009; Ikegami & Makino, 2009; Indran & Ikegami, 2012; Kortekaas, 2014; Kortekaas et al., 2011; Labeaud, 2010; Monath, 2013; Pepin et al., 2010). There are four main vaccines that are available but only three are available for use in RVFV endemic countries, including the Smithburn vaccine, Clone 13 vaccine, and a formalin-inactivated vaccine; the MP12 vaccine is also available but only for conditional use in the United States (Faburay et al., 2017; Mansfield et al., 2015). Although these vaccines are available, they all have limitations, creating the need for a safer, more immunogenic vaccine for

RVFV. Below is a table of the veterinary vaccines (**Table 1-2**) followed by a description of their limitations.

Table 1-3. List of veterinary vaccines available.

Vaccine name	Virus origin	Regimen	Licensed	Limitations
Smithburn live-attenuated vaccine	Mosquito, Uganda, 1948	Single dose	Yes	• Residual pathogenicity • Potential for teratogenic effects • Transmitted by mosquitoes
Inactivated vaccine	Field strains (South Africa and Egypt)	Requires booster and annual revaccination	Yes	• Booster requirement • Resource limited settings
Clone 13 live-attenuated vaccine	Human, 1974	Single dose	Yes (South Africa and Zimbabwe)	• Can cross placental barrier • Associated with fetal malformations
MP12 live-attenuated vaccine	Human strain ZH548 (Egypt, 1977)	Single dose	Conditionally licensed in the US	• Caused teratogenic effects during early stages of pregnancy

References: (Mansfield et al., 2015)

The live-attenuated Smithburn vaccine is the oldest and most widely used vaccine for controlling RVFV in South Africa (Mansfield et al., 2015). The vaccine was first produced as an avianized live-attenuated animal vaccine in South Africa via serial passage in a mouse brain and embryonated chicken egg (Grobbelaar et al., 2011). Since then, the vaccine stock has been propagated in BHK-21 cells (fibroblast cell line derived from baby hamster kidneys) for the formulation of a freeze-dried vaccine to use in livestock in South Africa, many other countries in Africa, and Saudi Arabia (Grobbelaar et al., 2011).

The appeal for using this vaccine is its relatively low cost and its ability to induce long-lasting immunity after a single dose (Mansfield et al., 2015). Despite the success with this vaccine, residual pathogenicity has been reported, creating the potential for teratogenic effects (Botros et al., 2006). Reports have claimed that administration of the vaccine to European breeds of cow led to abortion in 29% of pregnant animals (Botros et al., 2006). Therefore, the vaccine is only allowed for use in RVFV endemic areas. Further analysis has suggested that reassortment among RVFV isolates may occur in nature (Sall et al., 1999). If reassortment were to occur during an outbreak, there could be an increase in diversity of circulating RVFV strains (Ikegami, 2012). In addition, the Smithburn vaccine is said to play an important role in the persistence of the RVFV epidemic in Egypt because it has contaminated the environment and has been transmitted by mosquitoes (Kamal, 2009).

The formalin-inactivated vaccine was created by inactivating the RVFV Entebbe strain, isolated from a mosquito in Uganda using formalin (Mansfield et al., 2015). The vaccine is based on a previous formalin inactivated mosquito derived virus that was repeatedly passaged in mice (Mansfield et al., 2015) It is commercially available for veterinary use in South Africa. Another formalin-inactivated vaccine was propagated in BHK-21 cells and was used with an aluminum hydroxide adjuvant for veterinary use (Botros et al., 2006). Although theses inactivated vaccines induced a protective immune response, it required booster vaccinations, making it problematic because of the resource-limited settings it is used in (World Health Organization, 1983).

The Clone-13 vaccine is based on a naturally attenuated mutant, plaque derived clone that was isolated in the Central African Republic from a patient clinically infected with RVFV (Mansfield et al., 2015). The isolate had a natural deletion of most of the NSs gene and was attenuated in mice (Muller et al., 1995). In addition, it was highly immunogenic in pregnant ewes

without causing abortions or teratogenic effects (Dungu et al., 2010; Njenga et al., 2015; Swanepoel & Coetzer, 2004). Furthermore, the Clone 13 vaccine did not cause detectable viremia in vaccinated animals, thus minimizing the risk of vaccine virus transmission to mosquito vectors or to the fetus (Dungu et al., 2010). However, recent reports indicated that the Clone-13 vaccine could cross the ovine placental barrier and is associated with fetal malformations and stillbirths when administered in an overdose safety study to pregnant ewes in their first trimester (B. Makoschey et al., 2016). The vaccine has also caused neurological disease in mice when intranasally inoculated, meaning it may retain some virulence (Dodd et al., 2014).

Lastly, the live-attenuated MP12 vaccine was generated from the virulent strain ZH548 after 12 successive cell culture passages in the presence of 5-flurouracil (5FU) (Caplen et al., 1985). This was carried out by adding 5FU to the cell culture medium used with each passage of the virus (Caplen et al., 1985). When the virus was passaged in the presence of 5-fluorouracil, the rate of mutations increased, producing an attenuated virus. Later studies reported that the combination of multiple mutations fully attenuates the virus and prevents it from reverting to virulence (Ikegami et al., 2015). This is an important factor because if MP12 were to revert to virulence, it could cause abortions in vaccinated animals (Ikegami et al., 2015). The vaccine has been promising in both humans and animals and suitable immunogenicity in non-human primates following a high dose intravenous or aerosol challenge (Morrill et al., 1997; Morrill & Peters, 2011). However, the MP12 vaccine has caused teratogenic effects in ruminants during early pregnancy (Hunter et al., 2002).

Due to the limitations with the RVFV veterinary vaccines currently available, numerous vaccines to protect against RVFV are being developed and investigated. This dissertation work

evaluates a candidate live-attenuated vaccine for RVFV lacking the NSs and NSm genes in mice. To better understand the attenuation process, the genome structures for both CVV and RVFV are summarized below.

Genome Structure

The unifying characteristics of bunyaviruses include a bi- or tri-segmented, single-stranded, spherical, RNA genome of negative- or ambi-sense polarity that is 11-19 kb in size (Blitvich et al., 2018). The three genome segments are named according to their relative sizes, small (S), medium (M), and large (L), and code for various structural and non-structural proteins (**Figure 1-11**) (Albornoz et al., 2016). Although the genome is similar in its structure, the RNA segments vary in size among the different genera: the S segment ranges from 1 to 2.2 kb, the M segment from 3.5 to 6 kb, and the L segment from 6.3 to 12 kb (MacLachlan et al., 2017). The S segment encodes the nucleocapsid (N) protein and non-structural protein (NSs), the M segment encodes the viral glycoproteins (Gn and Gc) and non-structural protein (NSm), and the L segment contains the RNA-dependent RNA polymerase (RdRp) (Ariza et al., 2013; Hughes et al., 2020). The major genomic difference between CVV and RVFV is that the S segment within the CVV genome is negative sense whereas the S segment in the RVFV genome is ambi-sense (Bouloy & Weber, 2010; Dunn et al., 1994).

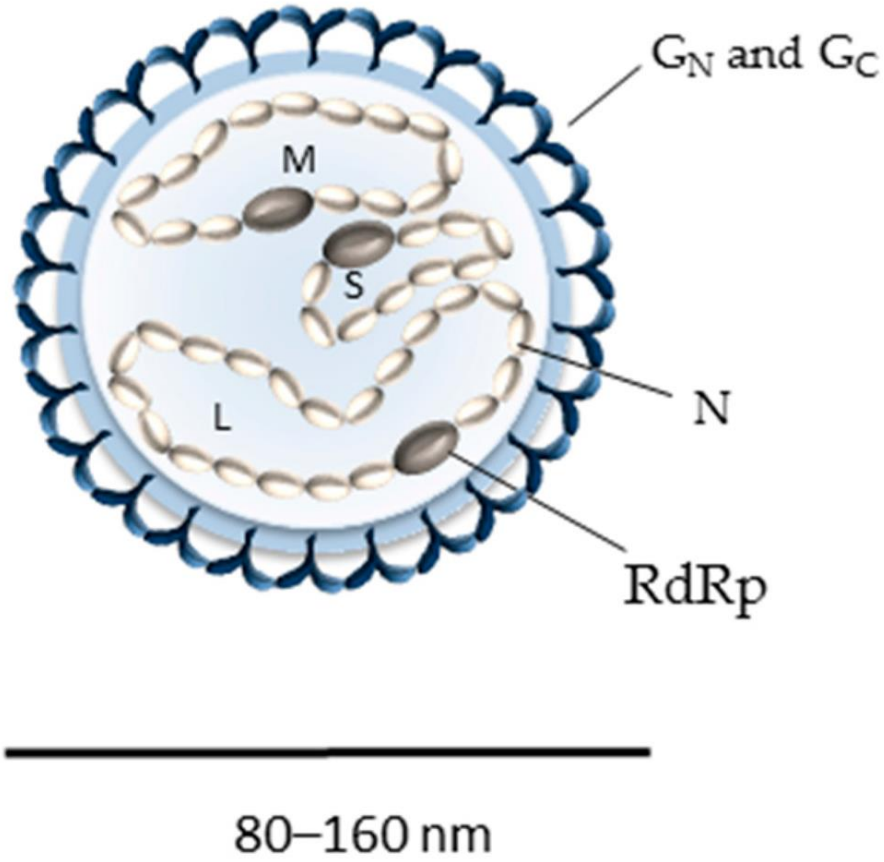

Figure 1-11. Schematic representation of a bunyavirus particle.

The three viral genomic segments are termed according to their size: S (small), M (medium) and L (large). Abbreviations: G_N: glycoprotein G_N; G_C: glycoprotein G_C; N: nucleoprotein; RdRp: RNA-dependent RNA polymerase. (Image used from (Albornoz et al., 2016); Published under the Creative Commons Attribution License).

The bunyavirus genome comprises four structural proteins: two surface glycoproteins (Gn and Gc), and two internal proteins, the N protein and the L protein. The N protein is the most abundant and highly immunogenic component of the RVF virion but has been shown to be highly conserved among members of the *Bunyaviridae* family (Gauliard et al., 2006; Skinner et al., 2022; Williams et al., 2011). This protein is also the main protein that is produced in infected cells (Elliott, 2014). The N protein can also interact with itself and the viral glycoproteins, Gn

and Gc, which are generated by co-translational cleavage and retained in the Golgi apparatus, the assembly site of bunyaviruses (Elliott, 2014; Fontana et al., 2008; Kuismanen et al., 1982; Murphy et al., 1973; Smith & Pifat, 1982; Spiegel et al., 2016). The genomic segments are encapsidated by the N protein and are associated with the viral RNA-dependent RNA polymerase to form ribonucleoprotein complexes (RNP) on each genomic segment and are contained in the lipid envelope of the particle (Elliott, 2014; Walter & Barr, 2011; Wichgers Schreur et al., 2018). The RNA-dependent RNA polymerase is responsible for the replication and transcription of the viral RNA in the cytoplasm of the cell (Jia & Gong, 2019; Jin & Elliott, 1991, 1992).

In addition to the structural proteins, there are non-structural proteins, NSs and NSm, encoded by many bunyaviruses within the S and M segment, respectively (Leventhal et al., 2021). The NSs protein is known to be a main virulence factor, counteracts the host innate immune response, and is mostly dispensable for virus replication (Blakqori et al., 2007; Kraatz et al., 2015; Weber et al., 2002). Little is known about the NSm protein's function however, it has been shown to be required for viral assembly (Lappin et al., 1994; Shi et al., 2006). **Table 1-3** summarizes the main functions carried out by each protein within the bunyavirus genome. Recent studies, as mentioned above, have been determining the involvement of the NSs and NSm proteins in virus pathogenicity to create virus vaccine candidates for bunyaviruses that are highly attenuated and immunogenic (Bird, Albarino, et al., 2008; Brennan et al., 2011; Dunlop et al., 2018; Kraatz et al., 2015; Szemiel et al., 2012). Therefore, this dissertation work studies the strategy to design a live-attenuated candidate vaccine for bunyaviruses, like CVV and RVFV, by deleting part of or all of the NSs and NSm genes.

Table 1-4. Summary of the major functions of CVV and RVFV proteins.

Protein type	Viral proteins	Major functions	References
Structural proteins	Nucleocapsid (N)	• Encapsidates the three genomic RNA segments to form ribonucleoprotein (RNP) complexes that associate with the RdRp.	(Elliott, 2014)
	Glycoprotein N (Gn)	• Used for entry into target cells and for assembly of progeny particles in infected cells.	(Spiegel et al., 2016)
	Glycoprotein C (Gc)	• Used for entry into target cells and for assembly of progeny particles in infected cells.	(Spiegel et al., 2016)
	L protein	• The RdRp, responsible for catalyzing transcription and replication.	(Elliott, 2014)
Nonstructural proteins	NSs	• Virulence factor • Accessory protein • May be involved in the inhibition of host cell protein synthesis. • Inhibits interferon induction	(A. Bridgen et al., 2001)
	NSm	• Interacts with the C terminus of Gn. • May be involved in the process of virus assembly and morphogenesis.	(Shi et al., 2006)

Bunyavirus replication

Although the replication cycle of most bunyaviruses is not fully defined, this section covers a basic overview of bunyavirus replication.

Virus Attachment and entry

Bunyavirus replication begins with attachment and cell entry. Cell entry involves interactions between the surface glycoproteins Gn and/or Gc and cell surface receptors, although the receptor (or receptors) remain to be identified (Elliott, 2014). Rift Valley fever virus attachment and entry into dermal dendritic cells at the site of initial infection (i.e. skin) has been

claimed to be mediated by dendritic cell-specific intercellular adhesion molecule 3- grabbing nonintegrin (DC-SIGN) (Lozach et al., 2011). More recently, studies suggest that DC-SIGN is unlikely to be a proteinaceous receptor (Ganaie et al., 2021). Instead, a low-density lipoprotein receptor-related protein 1 (mouse Lrp1/human LRP1) was identified as a novel proteinaceous host entry factor that is important for RVF infection across cell lines for multiple species (Ganaie et al., 2021). The exact receptors for CVV remain unknown; however, Gc has previously been suggested as the primary protein involved in attachment to both vertebrate and invertebrate cells in some studies while others have suggested that the smaller Gn protein of La Crosse encephalitis virus (LACV) functions as the attachment protein for mosquito cells (Hacker & Hardy, 1997; Ludwig et al., 1989; Ludwig et al., 1991; Plassmeyer et al., 2005). Understanding the functions of these two glycoproteins could advance our knowledge of how emerging CVV strains are transmitted.

After attachment, RVFV entry occurs by a dynamin-dependent caveola-mediated mechanism followed by membrane fusion by a pH dependent mechanism mediated by a Gc conformational change and uncoating in the late endosome (de Boer et al., 2012; Filone et al., 2006; Harmon et al., 2012). Caveola-mediated endocytosis is the infolding of the cells plasma membrane during endocytosis by pinching off and forming vesicles in the cytoplasm; the dynamin (hydrolase enzyme) acts as a pair of scissors to assist the newly formed vesicles by cleaving them from the plasma membrane (Henley et al., 1998). As for CVV, the entry process remains undefined. Studies have shown that OROV and LACV cell entry is likely to occur by clathrin mediated endocytosis though (Hollidge et al., 2012; Santos et al., 2008). Upon uptake, the bunyavirus particles are sorted into vesicles and transported to the appropriate endosomal

compartments for fusion and penetration into the cytosol; virus fusion proteins and uncoating also remain insufficiently characterized (Albornoz et al., 2016).

Genome replication and transcription

Since most bunyaviruses genomic RNA segments have a negative-sense polarity, the genomic RNA must be transcribed into positive-sense mRNAs. Therefore, once the virion is released in the cytosol, the RNPs, also known as the viral transcriptional machinery, transcribe their mRNAs to produce viral proteins (Ferron et al., 2017). However, the S segment in the RVFV genome is ambisense, where each segment is transcribed into mRNA and is replicated through a process involving the synthesis of complimentary RNA (cRNA) (Pepin et al., 2010). Since the RVFV S segment is ambisense, the proteins coded in the inverse polarity (positive) need to be transcribed using the positive sense viral cRNA as a template (Ferron et al., 2017). Therefore, the cRNA for RVFV represents the copy of the S ambisense segment and serves as a template for the synthesis of the NSs mRNA (Pepin et al., 2010). To initiate the transcription process for both CVV and RVFV, a unique mechanism that most negative-sense RNA viruses share called “cap-snatching” is carried out for mRNA. This mechanism is not well defined but has one study claimed that the viral L protein manages cap-binding and cleavage of the host mRNA downstream of the 5’ cap (Olschewski et al., 2020). The capped RNA fragment is then used as a primer for viral transcription (Olschewski et al., 2020). Additionally, synthesis of cRNA is initiated with 5’ nucleocapsid triphosphates for RVFV (Pepin et al., 2010).

Bunyavirus transcription is coupled with on-going translation suggesting that specific interactions may occur between the RNPs and the cellular translational machinery (Barr, 2007; Ferron et al., 2017). The mechanism that then shifts the polymerase from transcription to

replication is unclear, but the level of N protein in the cell is important, as sufficient N protein must be available to encapsidate nascent viral RNA to allow for replication to occur (Pinschewer et al., 2003).

Virus assembly and release

Following translation, the glycoproteins, Gn and Gc, are targeted to and localize in the Golgi complex using a signal in the transmembrane domain of Gn (Elliott, 2014). Other core virion proteins are recruited to the Golgi by unknown signals and mechanisms (Pepin et al., 2010). The progeny virions are then transported to the cell surface and released through exocytosis (Spiegel et al., 2016). Exocytosis is the final step and is the process by which cells move materials from within the cell into the extracellular fluid. A basic schematic for how negative-sense RNA virus replication occurs can be found below (**Figure 1-12**). It is important to understand how bunyaviruses replicate and which genes should be targeted, especially when trying to alter the genome to create vaccine viruses.

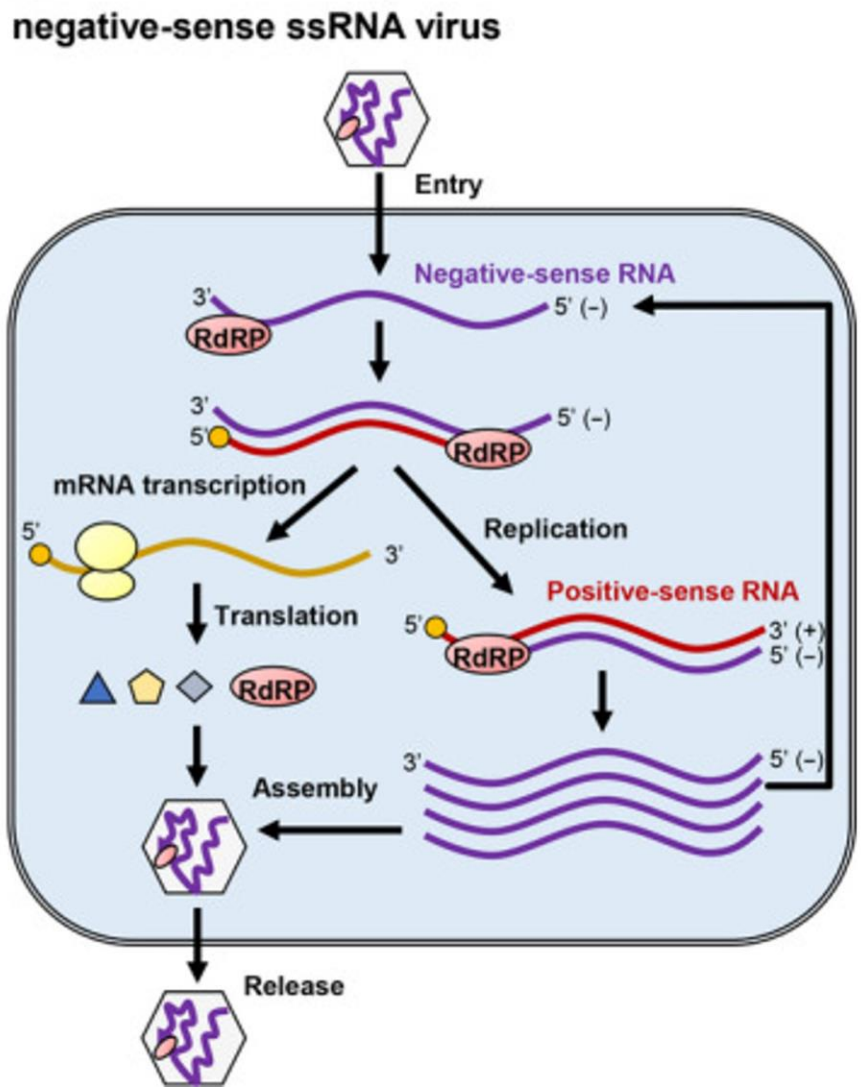

Figure 1-12. Life cycle of negative-sense single-stranded RNA (ssRNA) viruses.

Flowchart of RNA synthesis by RNA-dependent RNA polymerase (RdRP) of negative-sense ssRNA viruses. (Image modified from (Machitani et al., 2020); Published under the Creative Commons Attribution License.)

Vaccine Development

After describing the role of the NSs and NSm proteins above, the section below describes how these genes and their respective segments have been modified to create candidate vaccines.

Cache Valley virus candidate vaccine development

Initially, a reverse genetics system was developed for CVV (Dunlop et al., 2018). The goal was to attenuate the virus so it would elicit an immune response without the ability to revert to full virulence. All CVV viral segments were cloned and used to design specific primers for rapid amplification of cDNA ends (RACE) analysis (Dunlop et al., 2018). After the antigenomes were cloned, the sequences were confirmed using next generation sequencing (NGS). The NSs functions were then investigated by introducing mutations into the NSs open reading frame (ORF) to produce a recombinant virus that no longer expressed this protein (Dunlop et al., 2018). As shown in **Figure 1-13**, two methionines were replaced with threonines and two stop codons were introduced (Dunlop et al., 2018). Since NSs lies in an overlapping reading frame within the N ORF, the mutations were introduced in such a way as to prevent amino acid changes in the N ORF. The mutant virus, called rCVVdelNSs, was rescued by substituting the plasmids containing the wild-type S segments with those containing mutations in the NSs gene (Dunlop et al., 2018). To further attenuate CVV, the expression of the NSm protein was removed by deleting nucleotides 1039 – 1476 from the NSm gene (**Figure 1-14**), creating 2delCVV. 2delCVV was then characterized by plaque assays and growth in selected cell lines where it displayed slower growth than the wild-type CVV virus. Part of this dissertation work further evaluates the 2delCVV candidate vaccine in sheep and a mosquito vector for CVV.

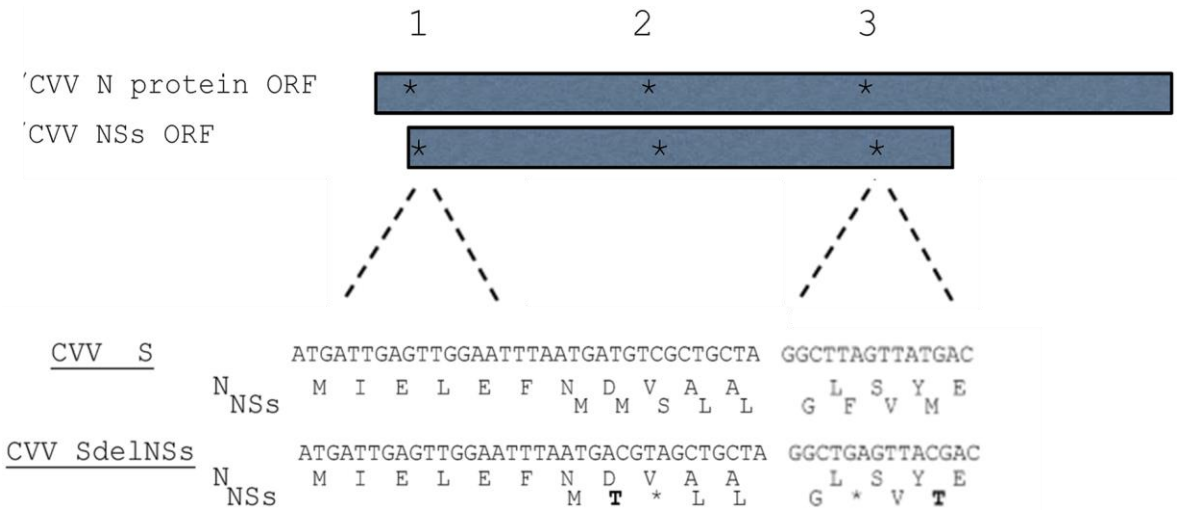

Figure 1-13. Design of CVV that does not express NSs.

Shown are sections of S segment for the N-termini of the N and overlapping NSs proteins. Mutations were added to disrupt the reading frames of the NSs proteins without changing the amino acid sequence of the overlapping N protein. Two methionines were changed to threonine (denoted in bold) and two stop codons introduced (denoted with an asterisk). Protein representation is not to scale. Image modified from (Dunlop et al., 2018); Published under the Creative Commons Attribution License.

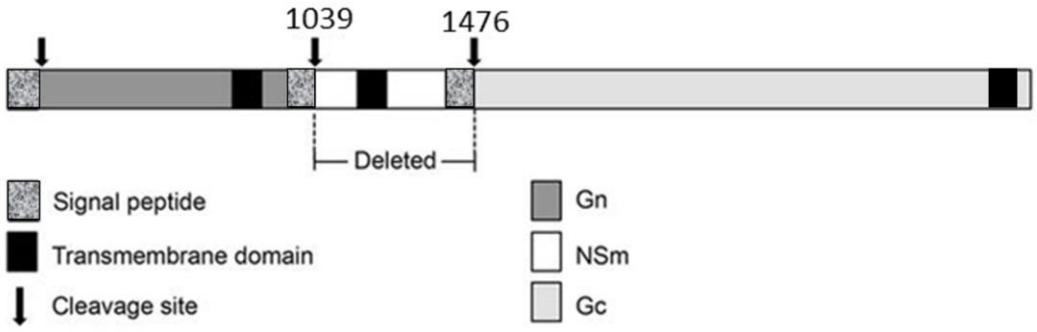

Figure 1-14. Schematic of the M segment showing Gn, NSm, and Gc regions.

The arrows depict where the cleavage occurs. The patterned boxes indicate the signal peptide, and the black boxes represent transmembrane domain. Nucleotides 1039-1476 were deleted in order to generate delNSm M segment. Image modified and used with permission from (Tilston-Lunel et al., 2015).

Rift Valley fever virus candidate vaccine development

There are multiple candidate live-attenuated vaccines for RVFV under development and all of them point to a central strategy for attenuation, achieved through genetic mutations of the virulence factor genes, NSs and NSm genes (Bird, Albarino, et al., 2008; Ikegami et al., 2006; Morrill et al., 2013; Morrill et al., 1997; Weingartl et al., 2014). The candidate live-attenuated vaccine for RVFV (r2segMP12) investigated in this dissertation is similar to others created but was developed using the attenuated MP12 strain as a backbone with the deletion of both virulence factors, NSs and NSm (Brennan et al., 2011). The other major difference, is this candidate live-attenuated vaccine was created to have a bi-segmented genome, rather than a tri-segmented genome, where the NSs coding sequence was replaced with the Gn and Gc precursors, creating a hybrid genomic S segment that maintained its ambi-sense coding strategy (**Figure 1-15**) (Brennan et al., 2011). Part of this dissertation work evaluated the immunogenicity of the r2segMP12 candidate vaccine in CD-1 mice.

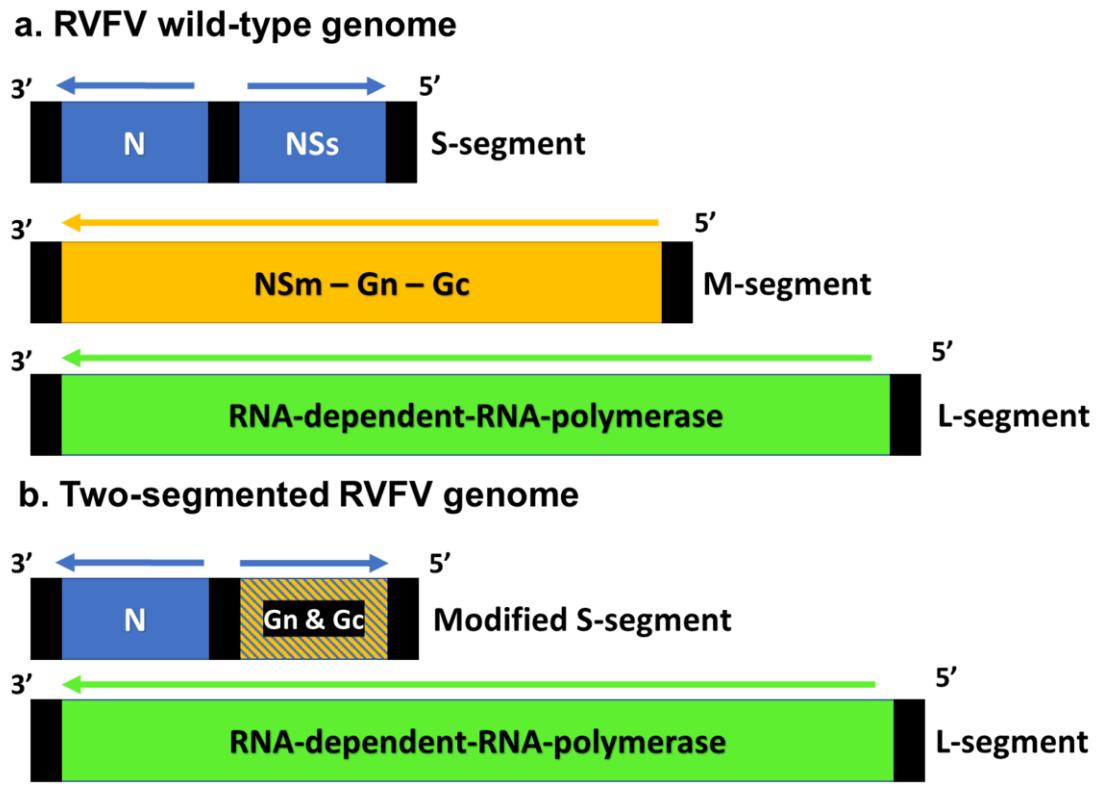

Figure 1-15. Schematic comparing the RVFV genome with the modified recombinant two-segmented RVFV genome.

Figure 1-15a represents the RVFV wild-type genome which includes the ambi-sense small (S-) segment consisting of the nucleocapsid (N) protein (negative-sense) and the non-structural protein, NSs (positive-sense); the negative-sense medium (M-) segment which contains the structural proteins, Gn and Gc, and the non-structural proteins, NSm and 78kD fusion protein; and the negative-sense large (L-) segment containing the L protein or RNA-dependent-RNA-polymerase (RdRp). Figure 1-15b represents the modified recombinant bi-segmented RVFV genome which only contains the S-segment and the L-segment. The NSs coding sequence has been replaced with the Gn and Gc precursors, maintaining the ambi-sense coding strategy, and the genome is lacking the authentic M RNA segment.

Despite the importance of these two viruses, there are no licensed vaccines available for either one. A previous study observed that live-attenuated vaccines are the most effective vaccines against arboviruses (Collins & Barrett, 2017). Hence this dissertation research studies

the virulence attenuation strategy by manipulating the genome of both viruses, as described above.

Justification for research and hypothesis

The overarching goal of this dissertation is to advance our knowledge and understanding of bunyaviruses while evaluating mitigation strategies for bunyaviruses. Two important bunyaviruses, CVV and RVFV, were investigated because they are both zoonotic, causing congenital abnormalities in ruminants and severe encephalitic disease in humans. Although CVV is endemic in the New World, it is likely that human cases are under-reported. This is most likely due to the insufficient evidence to identify the variety of clinical signs associated with the disease. Additionally, CVV has not always been a human pathogen, since most mosquitoes that feed on infected livestock do not usually feed on humans. Identifying new competent vectors can support the epizootic transmission of CVV thus, we can understand CVV pathogenesis and how it began causing disease in humans. There is also significant concern of RVFV being introduced into new territories which would create a problem for both animal and human public health. There is a wide distribution of competent vectors and susceptible ruminants creating a serious threat to the rest of the world. Understanding the biology of these two viruses will allow for the advancement of human health and well-being by furthering the development safe and immunogenic vaccines to prevent future outbreaks. We prove that the two attenuation strategies evaluated in this dissertation are effective in producing immunogenic candidate live-attenuated vaccines for orthobunyaviruses and phleboviruses. The knowledge gained from this dissertation can be directly translated to support the control of other emerging bunyaviruses.

The central hypothesis for this dissertation is that recombinant live-attenuated vaccines for CVV and RVFV induce neutralizing antibodies, are sufficiently attenuated in animals and are unable to replicate in medically important mosquitoes in North America.

Specific aims

Specific aim 1: Determine the vector competence of medically important mosquito species in North America for the transmission of CVV.

Medically important mosquito species found in North America were collected, colonized, and orally challenged with CVV to characterize the infection process. At 14 days post-infection (dpi), vector competence was determined by evaluating the presence or absence of viral RNA in saliva of infected mosquitoes. These data identified potential vectors responsible for the transmission of CVV from animal reservoirs to humans. The results are also important for the advancement of our knowledge for the ecology of CVV and its related regional subtypes in selected regions. As observed with other pathogenic arboviruses, the identification of competent vector species can be an important step in formulating mitigation strategies in the event of emergence.

Specific aim 2: Characterize a candidate live-attenuated vaccine (2delCVV) for CVV lacking the NSs and NSm genes.

A reverse genetics system was previously developed to generate a candidate live-attenuated vaccine for CVV that lacks both the NSs and NSm genes (Dunlop et al., 2018). This approach was used to produce a candidate live-attenuated vaccine named 2delCVV. The immunogenicity of the candidate 2delCVV live-attenuate vaccine was then assessed in sheep, the amplifying host for CVV, and compared to an autogenous binary-ethylenimine (BEI) inactivated

CVV candidate vaccine (BEI-CVV). Six-month-old sheep were subcutaneously immunized with either the candidate live-attenuated vaccine for CVV or an autogenous inactivated vaccine for CVV to compare the neutralizing antibody response induced by both vaccines. Neutralizing antibodies were evaluated because they are an important specific defense mechanism against viral invaders (Payne, 2017). The 2delCVV candidate vaccine not only produced a neutralizing antibody response through the duration of the study that could confer protection, but it also produced a slightly more robust neutralizing antibody response at the end of the study when compared to the autogenous vaccine. These data provide a basis for the development of immunogenic vaccines for other related bunyaviruses.

After evaluating the immunogenicity of 2delCVV in sheep, this vaccine was intrathoracically injected into *Ae. albopictus* mosquitoes. The purpose of this study was to compare the viral replication kinetics of the 2delCVV candidate vaccine to the CVV 6V633 wild-type strain in order to assess the potential of the vaccine virus to be transmitted by mosquitoes. Intrathoracic inoculation of a virus directly into the hemocoel bypasses the midgut barriers enabling the assessment of virus replication in the mosquito (Huang et al., 2020). The multiplication kinetics of the 2delCVV candidate vaccine was reduced by over one hundred folds when compared to the CVV 6V633 wild-type strain, demonstrating the restriction of the candidate vaccines replication.

<u>Specific aim 3</u>: Demonstrate the immunogenicity of a candidate live-attenuated RVFV vaccine in CD-1 mice.

RVFV is another important arbovirus that plays a significant role in human and veterinary health. Therefore, a two-segmented candidate live-attenuated vaccine for RVFV lacking the NSs and NSm genes was generated (Brennan et al., 2011). Before evaluating the

immunogenicity of the candidate live-attenuated vaccine (r2segMP12) in one of the amplifying hosts for RVFV, sheep, it was assessed for neutralizing antibodies in CD-1 mice. Mice were subcutaneously immunized with the r2segMP12 candidate vaccine to characterize the serum neutralizing activity. The conditionally licensed rMP12 parental strain vaccine was used to compare the superiority of the immune responses produced. These data generated indicate that the simultaneous deletion of the NSs and NSm genes in a two-segmented genome is a feasible approach to developing attenuated candidate vaccines for emerging bunyaviruses.

Together, the findings from these studies provided a better understanding of which medically important mosquitoes species in North America are potentially contributing to the epizootic and enzootic transmission cycle of CVV. In addition, previous reverse genetics systems were evaluated to determine if this technique could be a feasible approach to developing attenuated candidate vaccines for CVV, RVFV and other emerging bunyaviruses. The knowledge generated from these studies can aid in the development of effective countermeasures and prevention strategies against diseases that continue to cause severe disease in humans and/or animals resulting in substantial economic loss.

Chapter 2 - Investigation of virus-vector interactions between Cache Valley virus and medically important mosquito species

The experiments detailed in this chapter determine the vector competence of medically important mosquito species in North America for the transmission of CVV. This supports the hypothesis that some of these species may be involved in the transmission of CVV from viremic animals to humans. Mosquitoes were orally fed blood meals and the infection, dissemination, and transmission rates were determined for competency. The work in this chapter has been adapted from the manuscripts published by Springer Nature in *Parasites & Vectors* journal, available online at https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-019-3643-0 and at https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-018-3103-2.

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Introduction

Cache Valley virus (CVV) is a mosquito-borne orthobunyavirus, originally isolated from *Culiseta* (*Cs.*) *inornata* in Cache Valley, Utah, USA, during a routine testing of wild-caught mosquitoes for St. Louis encephalitis virus (SLEV) (Holden & Hess, 1959). Since then, CVV has been extensively found throughout North America and regarded as the most widely distributed member within the Bunyamwera serogroup in the New World (Andreadis et al., 2014; Blackmore & Grimstad, 1998; Blitvich, Lorono-Pino, et al., 2012; Burton et al., 1973; Campbell et al., 1989; Campbell et al., 1992; Eldridge et al., 1987; McLean et al., 1987; Meyers et al., 2015; Neitzel & Grimstad, 1991). Historically, CVV has been considered an important agricultural pathogen. While infection in adult sheep results in recovery and seroconversion, infection during pregnancy often leads to congenital malformations, stillbirths, and embryonic and fetal deaths (Chung, Livingston, Edwards, Crandell, et al., 1990; Chung, Livingston, Edwards, Gauer, et al., 1990). Enzootic transmission of CVV occurs among ungulates through bites of infected arthropod vectors. Experimental infections and data from serological surveys indicate that white-tailed deer (*Odocoileus virginianus*) are likely to be the amplification host and natural reservoir of CVV in nature (Blackmore & Grimstad, 1998; Campbell et al., 1989; Eldridge et al., 1987; Neitzel & Grimstad, 1991). Infections in humans are usually asymptomatic or associated with mild febrile illness. However, the public health significance of CVV has been increasingly recognized because of recent reports of severe human disease. Six human cases of CVV infection have been diagnosed in the United States since 1995 with more than one case ending in death (Campbell et al., 2006; Centers for Disease Control and Prevention, 2017; Nguyen et al., 2013; Sexton et al., 1997; Wilson et al., 2017; Y. Yang et al., 2018). In addition to the fatal cases reported, neuroinvasion of CVV has been observed during the acute phase of the

disease (Campbell et al., 2006; Nguyen et al., 2013; Sexton et al., 1997; Wilson et al., 2017; Y. Yang et al., 2018). Although the number of reported cases for CVV is low, seroprevalence is high and could cause introduction into naïve areas. Currently, there is no vaccine available for the control of CVV in animals or humans. The development of an effective vaccine can aid in the prevention of potentially fatal encephalitis in humans caused by the epizootic spillover and to control the enzootic transmission of CVV in animals.

As an emerging pathogen in the New World, serological surveys have demonstrated that humans can be exposed to CVV under various ecological conditions. The intensive transmission of CVV on the Eastern Shore of Maryland and Virginia, USA, coincided with the high seroprevalence rates among residents of Chincoteague Island, where saltwater marsh is the predominant mosquito habitat (Blitvich, Saiyasombat, et al., 2012; Buescher et al., 1970). More specifically, seroprevalence rates among individuals with exposure to farm and wild animals in the United States were reported to exceed 3% (Kosoy et al., 2016). Based on serology, CVV was demonstrated to occur in the urban and suburban environments in Latin America, where the majority of the mosquito infestation was associated with container-inhabiting mosquitoes, such as *Aedes* (*Ae.*) *aegypti* and *Ae. albopictus* (Almiron & Asis, 2003; Garcia-Rejon et al., 2011; Salomon-Grajales et al., 2012). Additionally, 5-7% of human serum samples collected from cities in Argentina were positive for neutralizing antibodies (Tauro et al., 2009). In the capital city of Mérida in the Yucatan State, Mexico, neutralizing antibodies against CVV can be found in 18% of individuals with febrile illness (Blitvich, Saiyasombat, et al., 2012). Similarly, the silent transmission of CVV has led up to 8% seroprevalence rates among residents of the Córodoba Province, Argentina (Tauro et al., 2009). It is possible that human infections may be higher than indicated by the low number of symptomatic cases. Despite the evidence suggesting

frequent transmission of CVV to humans, very little knowledge is known about the specific vectors responsible for the enzootic and epizootic transmission of CVV.

Although entomological surveys have been performed in the past, the objective of previously published studies was mainly to identify the species of enzootic vectors and their roles in the transmission and maintenance of CVV. It is well-accepted that multiple mosquito species in North America are competent for the transmission of CVV (Andreadis et al., 2014; F. Yang et al., 2018). In nature, CVV has been isolated from over 30 species of mosquitoes and more than ten mosquito species have been demonstrated to be competent vectors for CVV under laboratory conditions, some of those species include: *Cs. inornata*, *Anopheles* (*An.*) *quadrimaculatus*, *Coquillettidia* (*Cq.*) *perturbans*, *Ae. sollicitans*, *Ae. taeniorhynchus* and *Ae. japonicus* (Andreadis et al., 2014; Blackmore et al., 1998; Calisher et al., 1986; Centers for Disease Control and Prevention, 1985; F. Yang et al., 2018; Yuill & Thompson, 1970). However, the majority of competent vectors for CVV are not domestic or peridomestic species that can efficiently spread arboviruses among humans in urban and suburban areas. For instance, the two endemic vectors for CVV, *Anopheles quadrimaculatus* and *Cs. inornata* are not common in these urban and suburban areas (Eisen et al., 2008; Giordano et al., 2018). Other competent species, including *Ae. taeniorhynchus* and *Ae. sollicitans*, are normally found in saltwater marshes (Ritchie & Johnson, 1991; Shone et al., 2006). In addition, very few studies have been performed to identify the species responsible for the transmission of CVV from amplification hosts to humans: so-called bridge vectors. Endemic vector species of CVV do not show host preference for humans. For example, *Cs. inornata*, one of the principal vector species in nature, does not normally feed on humans (Anderson & Gallaway, 1987). Similarly, populations of *An. quadrimaculatus* and *Cq. perturbans* have been shown to predominantly feed on non-human

mammalian animals as observed with blood meal analyses conducted in several geographic regions (Apperson et al., 2002; Bingham et al., 2014; Cohen et al., 2009; Magnarelli, 1977; Molaei et al., 2008; Molaei et al., 2015; Robertson et al., 1993; Shepard et al., 2016). Therefore, the zoonotic transmission of CVV in specific ecological conditions may involve other mosquito species that show host preference for both animals and humans as observed with multiple zoonotic arboviruses.

Isolation of CVV from *Ae. albopictus* and *Ae. japonicus* is suggestive of the potential involvement of domestic and peridomestic *Aedes* species mosquitoes in the transmission of CVV from animals to humans (Andreadis et al., 2014; Armstrong et al., 2013). In the northeastern United States, both species have been found to be mammophillic, feeding on humans and white-tailed deer (Faraji et al., 2014; Goodman et al., 2018; Molaei et al., 2009). With the exception of *Ae. japonicus*, which has previously been investigated for its competence for CVV under laboratory conditions, the vectorial efficiency of domestic and peridomestic *Aedes* species for CVV remains largely undetermined (F. Yang et al., 2018). Although the percentage of CVV isolates obtained from *Culex* species mosquitoes is low in relation to the total number of available isolates, infection of CVV has been reported in at least three medically important species caught in the field, *Cx. pipiens, Cx. restuans*, and *Cx. tarsalis*. These observations warrant further investigation of whether or not *Culex* species mosquitoes can act as bridge vectors for the zoonotic transmission of CVV (Anderson et al., 2015; Andreadis et al., 2014; Calisher et al., 1986; Iversen et al., 1979). The potential importance of North American *Culex* species mosquitoes for the transmission of zoonotic arboviruses to humans has been well-established for several viruses including SLEV, Western equine encephalitis virus (WEEV), and West Nile virus (WNV) (Barnett, 1956; Goddard et al., 2002; Hammon & Reeves, 1943).

Therefore, determining the vector competence of medically important *Aedes* and *Culex* species mosquitoes for CVV is likely to provide information on the vector species responsible for its transmission from viremic animals to humans as well as the transmission of CVV to humans in the urban and suburban environment. Vector competence based on orally challenged mosquitoes identifies species that are able to transmit CVV in nature and exclude the candidate vector species that became a source of viral isolation due to recent engorgement.

In these studies, vector competence was determined with five species of mosquitoes, *Ae. aegypti, Ae. albopictus*, *Cx. pipiens*, *Cx. quinquefasciatus*, *Cx. tarsalis*, which were orally challenged with CVV to investigate the dynamics of infection, dissemination, and transmission. *Per os* infection was performed with 7-to-10-day-old female mosquitoes through oral exposure to viremic blood meals containing CVV. Mosquitoes were collected at 7- and 14-days post infection (dpi) for the assessment of infectious status. Forced salivation of immobilized mosquitoes was also performed at 14 dpi to determine the incidence of transmission.

Materials and methods

Virus and cell lines

The prototype 6V633 strain of CVV, originally isolated from infected *Cs. Inornata* in Cache Valley, Utah, in 1956 (Holden & Hess, 1959), was obtained from the collection in the laboratory of Dr. Richard M. Elliot (Watret et al., 1985). Sequences of all three genomic segments have been determined in a previously published study (GenBank accession numbers: KX100133.1, KX100134.1 and KX100135.1) (Groseth et al., 2017). This wild-type strain was generated by two passages in African green monkey kidney epithelial Vero76 cells and used in all oral challenge experiments for the determination of vector competence. Vero76 cells were

maintained in Leibovitz's L-15 media (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), 10% tryptose phosphate broth (Sigma-Aldrich, St. Louis, MO, USA), penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA), and L-glutamine (Thermo Fisher Scientific, Waltham, MA, USA) as previously described (Huang et al., 2015). The cells were cultured at 37°C and used for propagation of virus stocks, the processing of experimental samples, and titration of homogenized tissues as previously described (Huang et al., 2015).

Mosquitoes and *per os* infection

Five medically important mosquito species, *Ae. aegypti*, *Ae. albopictus*, *Cx. pipiens*, *Cx. tarsalis*, and *Cx. quinquefasciatus* were used in the experiments. *Per os* infection was performed with 7-10-day-old female *Ae. aegypti* Higgs white-eye strain (F>20), and *Ae. albopictus* (F_4), were derived from eggs collected from the city of Trenton, Mercer County, NJ, USA, in July 2016. Colonies of *Cx. pipiens* (F_8) and *Cx. quinquefasciatus* (F_{12}) were established from larvae and collected in Ewing Township, New Jersey and Vero Beach, Florida, as previously described (Huang et al., 2016). *Cx. tarsalis* used in this study originated from a collection in Kern County, California, USA (Eberle & Reisen, 1986).

The colonies were maintained by 10% sucrose solution under a 16:8 h light: dark photo regimen at 28°C. Mosquitoes were then deprived of water and sucrose 24 and 48h before *per os* infection, respectively. Viremic blood meals were prepared by mixing equal volumes of L-15 media containing CVV at 7.95 log of 50% tissue culture infectious dose ($TCID_{50}$)/ml and defibrinated sheep blood. Infectious blood meals were administered to *Aedes* species mosquitoes through a Hemotek membrane feeding system (Discovery Workshop, Lancashire, United

Kingdom) using previously described techniques (Huang et al., 2014). *Culex* species mosquitoes were exposed to infectious blood meals in gallon-size cartons through cotton pledgets at room temperature for 1 hour, as previously described (Huang et al., 2016; Vanlandingham et al., 2007). Control mosquitoes received blood meals containing a 1:1 volume mixture of L-15 media and defibrinated sheep blood.

Titers of viremic blood meals were determined by $TCID_{50}$ based titration of remaining blood meals aspirated from the cotton pledgets and individual blood feeders after the completion of each *per os* infection experiment, as previously described (Higgs et al., 2006). Engorged mosquitoes were cold-anesthetized and maintained in designated cartons at 28°C for the characterization of the infection process as previously described (Garcia-Rejon et al., 2011). Up to three engorged mosquitoes were collected at the end of the oral challenge experiment and titrated to confirm the ingestion of infectious viruses.

Mosquitoes were mechanically aspirated at 7- and 14- dpi and divided into two groups to characterize susceptibility to viral infection and dissemination in dissected mosquitoes and replication in whole carcasses. The disseminated form of infection was identified by the detection of infectious viruses in the secondary tissues (head, wings, and legs) of infected mosquitoes. The head, wings, and legs were dissected from the abdomen because they are infected with viruses disseminated from the midgut. The midgut is located inside the abdomen, which is where the infection of arboviruses is initially established. The second group consisted of whole carcasses used to determine the replication kinetics of CVV in infected mosquitoes. After collection, dissected and whole mosquitoes were frozen in individual 2ml Eppendorf tubes containing 2.8mm metal beads, 96 µl L-15 media, and 4 µl amphotericin.

Forced salivation

At 14 dpi, saliva was collected through forced salivation for 1 hour from each mosquito to determine the incidence of transmission (Huang et al., 2016; Vanlandingham et al., 2004). Mosquitoes were cold anesthetized by placing their cartons at 4°C until all mosquitoes were in the bottom of the carton. Once mosquitoes were transferred to a Petri dish, and forceps and a scalpel were used to remove the legs and wings. The mosquito's proboscis was then inserted into a capillary tube containing warm type B immersion oil (Cargille Laboratories Inc., Cedar Grove, NJ, USA) as previously described (Huang et al., 2015). After collecting saliva, the mosquitoes were removed and stored in previously labeled Eppendorf tubes. The oil containing the saliva was expelled from the capillary tube into a 1.5mL tube with 150 µl of L-15 media.

Detection of Cache Valley virus in mosquito tissues

The overall infection status of each individual mosquito was determined by the detection of infectious viruses in whole carcasses or dissected tissues using the $TCID_{50}$-based titration method with Vero76 cells, as previously described (Higgs et al., 2006; Huang et al., 2015). All samples were homogenized using a TissueLyser II apparatus (Qiagen, Germantown, MD) at 26 Hz for four minutes and titrated by $TCID_{50}$ with Vero76 cells (Higgs et al., 2006). Each well of a 96-well plate, except for the first column, received 90 µl of L-15 media. The empty wells were then filled with 100 µl of each mosquito sample, loaded in duplicate, followed by a 10-fold serial dilution. 100 µl of Vero76 cells was then placed in each well. Plates were sealed with parafilm and incubated for 7 days at 37°C. After incubation, individual wells were stained with 200 µl of amido black stain containing 1% amido black B10 suspended in 10% glacial acetic acid and 35% isopropanol aqueous solution at room temperature for 30 minutes. Each plate was then washed

with regular tap water and visually evaluated for cytopathic effect. Titers were calculated using the Reed-Muench method (Reed & Muench, 1938).

Infection rates were calculated using the percentage of infected mosquitoes among all mosquitoes tested at each time point. Dissemination rates were calculated by dividing the number of positive secondary tissues with the number of dissected mosquitoes that were infected with CVV.

Extraction with QIAamp Viral RNA Mini Kit

Extraction of viral RNA was performed with a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) before detecting the presence of CVV in the saliva of infected mosquitoes. Five hundred and sixty µl of prepared Buffer AVL containing carrier RNA was pipetted into a 2mL microcentrifuge tube. One hundred and forty µl of saliva sample was added to the microcentrifuge tube and vortexed for 15 seconds. The tubes were incubated for 10 minutes at room temperature and then briefly centrifuged to remove the drops from inside lid. Five hundred and sixty µl of ethanol was added to the sample and mixed by vortexing for 15 seconds before briefly spun in the centrifuge again. Six hundred and thirty µl of the solution from the previous step was carefully added to the QIAamp Mini column in a 2mL collection tube. Once the cap was closed, the tubes were centrifuged at 8,000 rpm for one minute. The collection tube was then emptied, and the remainder of the mixture was added to the column. The QIAamp Mini column was placed into a new 2mL collection tube and the tube containing the filtrate was discarded. Five hundred µl of Buffer AW1 was added and the tubes were centrifuged at 8,000 rpm for one minute. The QIAamp Mini column was placed into a new 2mL collection tube and the tube containing the filtrate was discarded. Five hundred µl of Buffer AW2 was added and the tubes

were centrifuged for three minutes at 13,000 rmp (maximum speed). The QIAamp Mini column was removed from the tube with the filtrate and placed into a new 2mL collection tube. Sixty µl of molecular grade water was added, the tubes were incubated for one minute at room temperature, and then centrifuged for one minute at 8,000 rmp. The QIAamp Mini column was removed and discarded. The extractions were stored in RNAse free microcentrifuge tubes at -80°C.

Detection of Cache Valley virus in saliva by reverse-transcribed nested polymerase chain reaction

The presence of viral genome in the saliva of orally challenged mosquitoes was demonstrated by reverse-transcriptase nested polymerase chain reaction (nested RT-PCR). Complementary DNA (cDNA) was produced by reverse transcription of Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and then synthesized by the gene specific primer CV-Mex-R (5′-GACGTCTGTTAAGAAGCAAGTTGAGTTT-3′). The cDNA was then amplified using a nested approach with Platinum Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA was amplified based on the following previously published primers (first primer set: CV-Mex-F: 5′-GCACTCTGGCAGGCAGGA-3′ and CV-Mex-R: 5′-GACGTCTGTTAAGAAGCAAGTTGAGTTT-3′; second primer set: CV-G1-F: 5′-CCAATGCAATTCAGGGCAGT-3′ and CV-G1-R: 5′-TGAGTCACCACATGCTGTAAGGT-3′) (Wang et al., 2009). The outer primer set was designed to target nucleotide positions between 2220 and 2520 of the G1 gene encoded within the medium (M) genome segment. Amplicons derived from the outer primer set were amplified by the inner primer set targeting the nucleotide positions between 2246 and 2358 of the G1 gene. All amplicons were separated and visualized

by electrophoresis on 4% agarose gels. To generate the cDNA by reverse transcription, 1 µl of 2 µM CV-Mex-R primer, 1 µl of 10 mM dNTP mix, 3 µl of H2O, and 8 µl of RNA were combined into 0.2 mL PCR tubes. The tubes were heated for five minutes, then the thermocycler was paused, and the tubes were placed on ice for one minute. Four µl of 5x FS Buffer, 1 µl of DTT, 1 µl of RNaseOUT, and 1 µl of Superscript III were added. For the first amplicon, 2.5 µl of template cDNA, 2.5 µl of 10X PCR buffer, 0.75 µl of $MgCl_2$, 0.5 µl of 10 mM dNTP mix, 0.5 µl of 10 µM CV-MEX-R, 0.5 µl of 10 µM CV-MEX-F, 0.1 µl of Taq polymerase, and 17.65 µl of molecular grade water were combined. For the second amplicon, 2.5 µl of template cDNA, 2.5 µl of 10X PCR buffer, 0.75 µl of $MgCl_2$, 0.5 µl of 10 mM dNTP mix, 0.5 µl of 10 µM CV-G1-R, 0.5 µl of 10 µM CV-G1-F, 0.1 µl of Taq polymerase, and 17.65 µl of molecular grade water were combined. Reactions were performed using a Bio-Rad Thermal cycler (Bio-Rad, Hercules, CA) with cycling parameters for reverse transcription at 65°C for five minutes, ice for one minute while assembling reactions, 55°C for 60 minutes, 70°C for 15 minutes, and 4°C for five minutes. For PCR the cycling parameters was 95°C for ten minutes, 45 cycles of 95°C for 15 seconds, and 60°C for one minute.

For creation of a 4% gel, four grams of agarose was measure. The agarose powder was mixed with 100 mL of 1xTAE and microwaved until completely dissolved. Once the agarose was cooled down, it was poured into a gel tray with a well comb and allowed to solidify. The agarose gel was place into the electrophoresis unit and filled with 1xTAE until the gel was completely covered. Five µl of sample and 1 µl of red marker was loaded into the wells of the gel, followed by a 100 base pair marker. The gel was run at 125V for 40 minutes. Once finished, the gel was removed from the electrophoresis unit, placed on a UV light, and observed.

Statistical analysis

All statistical analyses were conducted using GraphPad (San Diego, CA, USA), SigmaPlot (San Jose, CA, USA), and Excel software (Redmond, WA, USA). Differences in the infection, dissemination, and transmission rates were determined using Chi-square or Fisher's exact test, depending on the sample sizes in the contingency tables. Titers of infected mosquitoes were compared with Mann-Whitney rank sum test between two groups when normal distribution is not observed or Student's t-test when infectious titers follow normal distribution.

Results

Infection and dissemination of CVV

Five species of medically important mosquito species in the *Aedes* and *Culex* genera showed variations in susceptibility to CVV infection through oral exposure. As summarized in **Table 2-1**, the establishment of infection in the *Culex* species mosquitoes was only observed in *Cx. tarsalis*; whereas *Cx. pipiens* and *Cx. quinquefasciatus* were refractory to CVV. Infectious viruses of CVV were not detected among 28 and 27 *Cx. pipiens* collected at 7 and 14 dpi, respectively. Similarly, *Cx. quinquefasciatus* mosquitoes collected at 7 (n = 14) and 14 (n = 18) dpi did not show a detectable level of infectious viruses. As demonstrated by the isolation of infectious viruses in homogenized mosquito tissues, there was no distinguishable difference in the infection rates of CVV in *Cx. tarsalis* at 7 (81.8%, 18/22) and 14 (82.6%, 19/23) dpi (p = 1.00). The dissemination rate of CVV in infected *Cx. tarsalis* showed a significant increase from 72.7% (8/11) at 7 dpi to 100.0% (9/9) at 14 dpi (Fisher's exact test: $p< 0.05$), presumably due to the continuous viral replication in permissive tissue. However, there was no demonstrable difference in the average titer of infected whole mosquitoes at 7 (5.41 ± 2.06 $\log TCID_{50}$/ml, n =

7) and 14 (5.47 ± 1.07 $logTCID_{50}$/ml, *n* = 10) dpi as shown in **Figure 2-1**. Similarly, there were no significant differences in the infectious titers of CVV present in the dissected abdomen section (7 dpi: 4.23 ± 1.49 $logTCID_{50}$/ml, *n* = 11; 14 dpi: 4.91 ± 0.51 $logTCID_{50}$/ml, *n* = 8) and secondary tissues (7 dpi: 5.03±1.48 $logTCID_{50}$/ml, *n* = 8; 14 dpi: 5.09 ± 1.48 $logTCID_{50}$/ml, *n* = 9). Detection of infectious viruses in mosquitoes collected at 7 and 14 dpi indicated that *Cx. tarsalis* is highly susceptible to CVV through oral challenge and subsequently supports viral replication. *Cx. pipiens* and *Cx. quinquefasciatus* are highly refractory to CVV.

Table 2-1. Summary of infection and dissemination rates in *Culex* species mosquitoes orally challenged with Cache Valley virus.

Mosquito species	7 dpi		14 dpi	
	Infection rate (%)[a]	Dissemination rate (%)[b]	Infection rate (%)[a]	Dissemination rate (%)[b]
Cx. tarsalis	81.8 (18/22)	72.7 (8/11)	82.6 (19/23)	100.0 (9/9)
Cx. pipiens	0.0 (0/28)	na	0.0 (0/27)	na
Cx. quinquefasciatus	0.0 (0/14)	na	0.0 (0/18)	na

Abbreviation: *na* not available. [a]Infection rates were derived from the percentage of infected mosquitoes among all the mosquitoes tested at each time-point (numbers in parentheses). [b]Dissemination rates were calculated by dividing the numbers of mosquitoes containing positive secondary tissues with the number of dissected mosquitoes that were infected by CVV (numbers in parentheses).

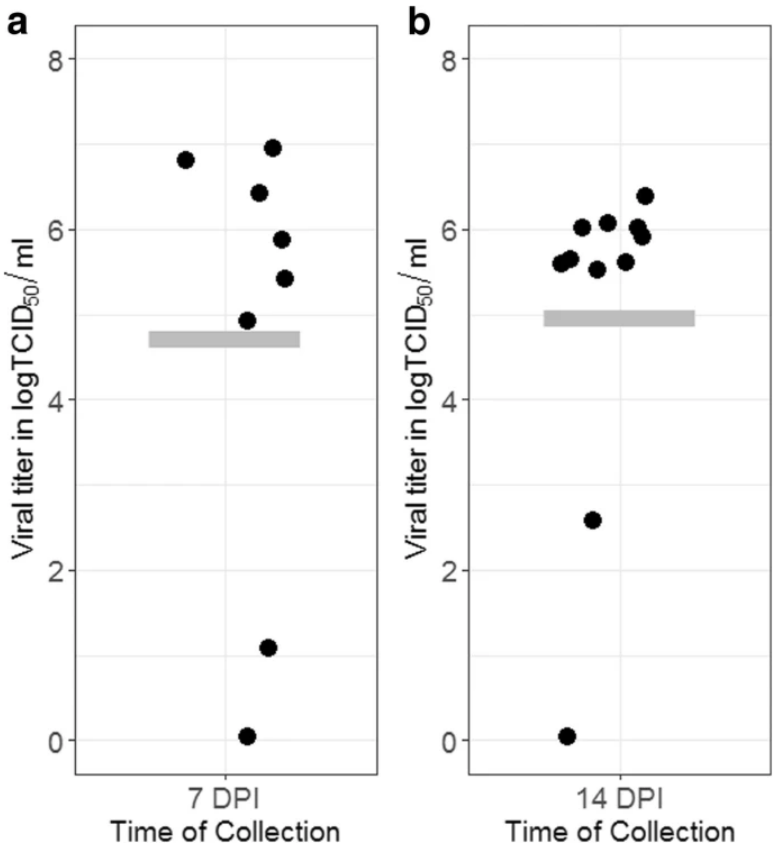

Figure 2-1. Titers of *Cx. tarsalis* infected with CVV.

(a) Titers at 7 dpi, (b) Titers at 14 dpi. The horizontal bar represents the average titer of whole mosquitoes.

Oral challenge with CVV led to the establishment of infection in both *Ae. aegypti* and *Ae. albopictus* as summarized in **Table 2-2**. the significantly higher infection rates were observed in *Ae. albopictus* at both 7 dpi [*Ae. albopictus:* 69.2% (45/65) *vs Ae. aegypti*:15.2% (10/66), Chi-square test: $\chi^2 = 37.13$, $df = 1$, $p < 0.001$] and 14 dpi [*Ae. albopictus:* 56.5% (26/46) *vs Ae. aegypti*: 11.0% (9/82), Chi-square test: $\chi^2 = 28.52$, $df = 1$, $p < 0.001$], indicating higher susceptibility of *Ae. albopictus* to CVV than *Ae. aegypti*. Consistent with the differences in susceptibility, *Ae. albopictus* supported more rapid replication of CVV as demonstrated by significantly higher average titers of whole mosquitoes collected at 7 dpi [*Ae.*

albopictus (average titer ± standard deviation): 5.0 ± 2.2 $logTCID_{50}$/ml *vs Ae. aegypti* (average titer ± standard deviation): 3.1 ± 2.7 $logTCID_{50}$/ml; t-test: $t = 1.713$, $df = 19$; $p = 0.02$] (**Figure 2-2**). Although there was no significant difference in titers of infected mosquitoes at 14 dpi [*Ae. albopictus* (median titer): 6.0 $logTCID_{50}$/ml *vs Ae. aegypti* (median titer): 5.5 $logTCID_{50}$/ml; Mann–Whitney test: $U = 22$, $p = 0.55$]. While significant differences in susceptibility and replication kinetics were observed, the incidence of disseminated infection was indistinguishable at 7 dpi [*Ae. albopictus:* 83.3% (25/30) *vs Ae. aegypti*: 100% (4/4), Fisher's exact test: $\chi^2 = 37.13$, $df = 1$, $p = 1.00$] and 14 dpi [*Ae. albopictus*: 100% (12/12) *vs Ae. aegypti*: 100% (5/5)]. Our results indicate that infection with CVV can be established in both *Ae. albopictus* and *Ae. aegypti* through the ingestion of viremic blood meals.

Table 2-2. Infection and transmission of Cache Valley virus by *Aedes albopictus* and *Aedes aegypti* mosquitoes.

Mosquito species	0 dpi	7 dpi		14 dpi		
	Average titers of engorged mosquitoes ($logTCID_{50}$/ml)	Infection rate (%)[ab]	Dissemination rate (%)[c]	Infection rate (%)[ab]	Dissemination rate (%)[c]	Transmission rate (%)[d]
Ae. albopictus	3.7 ± 0.6 (*n* = 11)	69.2 (45/65)	83.3 (25/30)	56.5 (26/46)	100.0 (12/12)	29.6 (8/27)
Ae. aegypti	4.0 ± 0.8 (*n*=18)	15.2 (10/66)	100 (4/4)	11.0 (9/82)	100 (5/5)	30.0 (3/10)

[a]The infection rate of CVV at 7 and 14 dpi was determined by the isolation of infectious viruses in tissues of dissected mosquitoes or carcasses of whole mosquitoes using Vero76 cells. [b]Significant differences between *Ae. albopictus and Ae. aegypti* were detected using Chi-square test. [c]The dissemination rate of CVV at 7 and 14 dpi was calculated based on the detection of infectious viruses in secondary tissues (head, wings and legs) of dissected mosquitoes, which were infected with CVV. [d]The transmission rate of CVV was determined by the incidence of positive detection of viral RNA among saliva of infected mosquitoes using nested RT-PCR.

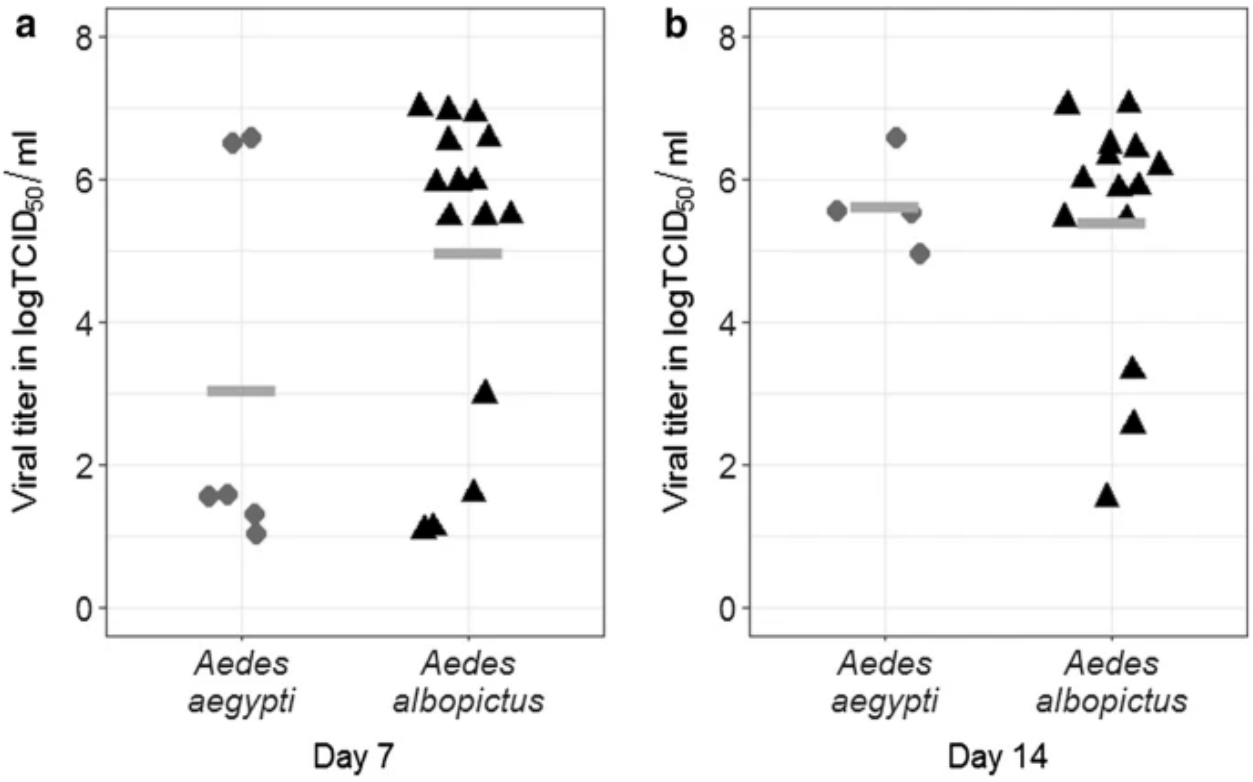

Figure 2-2. Infectious titers of whole mosquitoes infected with CVV.

(a) Viral titers at 7 dpi, (b) viral titers at 14 dpi. Titers of infected *Ae, aegypti* and *Ae. albopictus* are shown as circles and triangles, respectively. The grey solid line represents the average titer of each species at 7- and 14- dpi.

Detection of viral RNA in mosquito saliva

With the high infection and dissemination rates observed in *Cx. tarsalis* orally challenged with CVV, saliva obtained through forced salivation of individual mosquitoes at 14 dpi was assayed for the presence of the M segment of viral genome through nested RT-PCR. As anticipated, none of the saliva samples collected from *Cx. pipiens* (n = 27) and *Cx. quinquefasciatus* (n = 18) showed a detectable level of viral genome. Viral RNA of CVV was detected from 31.6% (6/19) of infected *Cx. tarsalis*. These results demonstrate that CVV is able to develop disseminated infection in *Cx. tarsalis*, which can subsequently be competent for its transmission.

At 14 dpi, viral RNA was detected in saliva collected from 29.6% (8/27) of infected *Ae. albopictus*. Similarly, 30.0% (3/10) of infected *Ae. aegypti* also showed a positive detection of viral RNA in the saliva. The presence of viral RNA following oral exposure to CVV indicates that both species are competent vectors for CVV. Although the transmission rate for both species was approximately 30%, the higher infection rates for *Ae. albopictus*, as compared to *Ae. aegypti*, indicate that there could potentially be more infected *Ae. albopictus* involved in the overall transmission of CVV.

Discussion

The results of our study demonstrated that *Ae. aegypti*, *Ae. albopictus*, and *Cx. tarsalis* are susceptible to CVV. While the differential susceptibility suggests there can potentially be a difference in the likelihood of the three species in vectoring CVV to humans in nature, especially in the Southern United States and Latin America, the confirmation of *Cx. tarsalis* and *Ae. albopictus* as competent vectors is of high public and veterinary health importance. Previous analyses of the blood-feeding behavior of *Cx. tarsalis* further supports its potential role in maintaining enzootic transmission of CVV, especially in the Midwestern states of the USA. In addition, the relatively high competence of *Ae. albopictus* demonstrated in this study, and large numbers of isolates recovered from nature, suggest that this species may be actively involved in the enzootic and epizootic transmission of CVV in regions where viremic vertebrate hosts and humans coexist (Armstrong et al., 2013). In two independent studies, *Cx. tarsalis* from North Dakota and Minnesota, where CVV is endemic, showed relatively high frequencies of feeding on white-tailed deer, the known amplification host of CVV in nature (Blackmore & Grimstad, 1998; Friesen & Johnson, 2013; Mehus & Vaughan, 2013). In addition to its role as an enzootic vector,

the established role of *Cx. tarsalis* as a vector for SLEV, WNV, and WEEV in western USA and its documented feeing on humans, further supports the hypothesis that it may be involved in the transmission of CVV from viremic animals to humans (Goddard et al., 2002; Hammon & Reeves, 1943).

Interestingly, detection of CVV in *Ae. albopictus* coincides with the dispersal of lineage two viruses from southern Mexico to the northeastern USA. All CVV isolates from *Ae. albopictus* in northeastern USA have also been demonstrated to cluster under the same lineage two. These findings warrant further investigation whether the species also contributed to the emergence of the new genetic lineage in North and Central America (Armstrong et al., 2013; Armstrong et al., 2015). As the distribution of *Ae. aegypti* in North America has just exceeded 33°N latitude between 2011 and 2014 and the introduction of *Ae. albopictus* did not take place until the 1980s, the high prevalence of neutralizing antibodies against CVV in the eastern shore of Maryland and Virginia observed in the 1960s was unlikely to be caused by transmission vectored by the two competent species identified in this study (Buescher et al., 1970). However, the involvement of other vectors known for zoonotic transmission of arboviruses including *Ae. sollicitans* and *Ae. taeniorhynchus*, may be a more plausible explanation.

Although there is variation in the frequency of feeding on humans, engorgement from human blood has been repeatedly observed in multiple populations of *Cx. tarsalis* in nature (Campbell et al., 2013; Friesen & Johnson, 2013; Mehus & Vaughan, 2013; Molaei et al., 2008). Host preference is, in part, determined by changes in host availability, suggesting that the contact rate with humans may depend on the diversity of other potential hosts (Kent et al., 2009; Thiemann et al., 2011). As a species that has evolved to hibernate and has been shown to support the overwintering of arboviruses such as SLEV, WNV and WEEV (Reisen, Fang, Lothrop, et al.,

2006; Reisen et al., 2002), our findings also highlight the need to further investigate the ecology of *Cx. tarsalis* and its involvement in the overwintering maintenance of CVV in nature. Similar to other orthobunyaviruses, vertical transmission has been demonstrated to be a likely overwintering mechanism for CVV (Hayles & Lversen, 1980). For instance, 2.9 to 3.3% of experimentally infected *Cs. Inornata* transovarially transmitted CVV to both male and female progeny. Further investigations in the detection of CVV in overwintering populations of *Cx. tarsalis* in nature will provide much needed understanding of the maintenance of CVV.

As an invasive species, the increasing infestation of *Ae. albopictus*, and its high competence for CVV, also raised an interesting question: whether or not the introduction and potential spread of this species will change the epidemiology of CVV and other agriculturally important arboviruses in different regions of the Americas (Kraemer et al., 2019)? Increased autochthonous transmission of various human and zoonotic arboviruses vectored by *Ae. albopictus* has established its importance as a species that impacts human public health (Vanlandingham et al., 2016). However, much less is known regarding its importance with respect to animal health. It will be of great human and animal health importance to further define the role of *Ae. albopictus* in vectoring CVV among animal reservoirs and humans, especially those located in infested areas. The findings may be helpful in defining the health risk associated with CVV infection, which remains largely unknown.

Based on our results, *Ae. aegypti* is likely to have limited contribution to transmission of CVV in nature because of the low susceptibility demonstrated in this study. The use of *Ae. aegypti* Higgs white-eye strain, a colonized strain derived from the Puerto Rican RexD colony and selected based on the high competence of a variety of arboviruses including several orthobunyaviruses, further support our conclusion (Hughes et al., 2006). Although the species

can be competent for CVV under laboratory conditions, a large number of infected mosquitoes may be required for the intensive transmission that leads to the observed occurrence of a high seroprevalence rate. Entomological surveys have demonstrated that naturally occurring infection from *Ae. aegypti* with CVV is a rare event (Farfan-Ale et al., 2010). Therefore, *Ae. aegypti* is unlikely to serve as an important urban vector responsible for frequent human exposures to CVV and its related subtypes. To the best of our knowledge, infestation of *Ae. albopictus* has not yet been reported in the Córodoba province, Argentina. The population of *Ae. aegypti* has been known to be involved in the transmission of arboviruses in the region but should not contribute to the transmission of CVV to humans (Estallo et al., 2014; Rotela et al., 2017).

Collectively, available evidence suggests that high prevalence rates of human neutralizing antibodies against CVV in Latin America may involve transmission by other mammophillic domestic and peridomestic mosquito species. Identifying such species will be particularly important for the advancement of our knowledge for the ecology of CVV and other regional subtypes in selected regions in Latin America. Although the number of reported neurotropic cases of CVV remains low, the advancement of virological and molecular biological techniques has led to the identification of variants or subtypes of CVV that are responsible for human diseases throughout the New World (Calisher et al., 1988). In 1985, the isolation of Fort Sherman virus was made from an American soldier in Panama who developed fever and an erythematous pharynx at the acute phase of infection (Mangiafico et al., 1988). Similarly, Maguari virus, another orthobunyavirus closely related to CVV, has been continuously found in multiple Latin American countries causing febrile illness in humans (Groseth et al., 2017). It remains unclear if other pathogenic orthobunyaviruses closely related to CVV can also utilize *Cx. tarsalis* or *Ae. albopictus* for transmission or maintenance. As observed with many other

pathogenic arboviruses, identification of competent vector species can be an important step in formulating control strategies in the event of emergence.

Chapter 3 - Evaluating a candidate live-attenuated vaccine for CVV lacking the NSs and NSm genes

The experiments detailed in this chapter focus on characterizing a candidate live-attenuated vaccine (2delCVV) for CVV lacking the NSs and NSm genes in sheep and mosquitoes. Initially, the immunogenicity of the candidate live-attenuated vaccine was compared to an autogenous binary-ethylenimine (BEI) inactivated vaccine for CVV in sheep. After demonstrating the development of neutralizing antibodies in sheep immunized with 2delCVV, the replication kinetics of this candidate vaccine was determined in *Ae. albopictus* and compared to the wild-type CVV strain. The results demonstrated the effect of the attenuated virus within a competent vector species. These data provide information for further developing immunogenic vaccines for CVV and other related bunyaviruses. Part of the work in this chapter has been submitted for peer review and publication in *Viral Immunology* and some of this work has been submitted for peer review and publication in *Vector-Borne and Zoonotic Diseases*.

Introduction

Cache Valley virus (CVV) is an important agricultural pathogen causing embryonic and fetal death, neonatal malformations, and abortions in ruminants, especially sheep (Edwards et al., 1989). Cache Valley virus has also recently been recognized for its expansion as a zoonotic pathogen. Despite its importance in the livestock industry and its spread to human hosts, there are no licensed vaccines commercially available to prevent or control CVV. With the increased emergence of bunyaviruses with human and veterinary importance, there have been significant efforts dedicated to the development of bunyavirus vaccines.

Most live-attenuated arbovirus vaccines with excellent safety profiles have been shown to lose the ability to infect and replicate in mosquitoes which is critical when preventing an outbreak (Monath et al., 2020). For example, two highly effective live attenuated vaccines for arboviruses, e.g., yellow fever 17D vaccine and Japanese encephalitis SA14-14-2 vaccine, are unable to replicate and disseminate in mosquitoes (Chen & Beaty, 1982; Danet et al., 2019). In contrast, vaccine strains that did not reach a safe level of attenuation can often infect, multiply, and disseminate in mosquitoes. For example, the live-attenuated vaccine TC-83 for Venezuelan equine encephalitis was capable of infecting biting mosquitoes following equine vaccination (Pedersen et al., 1972). This generated a widespread circulation of the mutant virus in agricultural areas, causing an epidemic. The use of the live-attenuated Smithburn vaccine during RVFV outbreaks in Egypt may have resulted in the spread of RVFV instead of eradicating the virus (Kamal, 2009). Calves and pregnant cows were suffering illness, mortalities, and abortion storms following vaccination during these outbreaks, possibly due to post-vaccinal reactions to the Smithburn vaccine (Ahmed Kamal, 2011). This lead to the belief that the vaccine is unsafe, especially during mosquito breeding season, when the vaccine virus could revert to virulence and be transmitted by mosquitoes (Ahmed Kamal, 2011).

To date, the virulence phenotype of multiple orthobunyaviruses, such as Bunyamwera virus (BUNV) and Schmallenberg virus (SBV), has been attenuated by the deliberate removal of virulence factors in the viral genome (Kraatz et al., 2015; Szemiel et al., 2012). The orthobunyavirus genome consists of three negative-sense RNA segments, small (S), medium (M), and large (L), which code for various structural and non-structural proteins (Hughes et al., 2020). Orthobunyaviruses have two known virulence factors, the NSs gene encoded in the S segment and the NSm gene encoded in the M segment (Elliott, 2014). Deletion of either

virulence factors is sufficient for virulence attenuation, as demonstrated with BUNV, the prototype orthobunyavirus (A. Bridgen et al., 2001; Szemiel et al., 2012). Additionally, the simultaneous deletion of NSs and NSm genes fully attenuated the virulence phenotype of SBV, an emerging orthobunyavirus, in immunocompromised mice (Kraatz et al., 2015). The functions of the NSs and NSm proteins have become elucidated in more detail recently to study the attenuation process of viruses for the development of vaccines.

The function of the NSs protein have been evaluated in several orthobunyaviruses, including Cache Valley virus (CVV), Kairi virus (KRIV), Oropouche virus (OROV), Akabane virus (AKAV), BUNV, SBV, and La Crosse encephalitis virus (LACV) (Blakqori et al., 2007; A. Bridgen et al., 2001; Dunlop et al., 2018; Ishihara et al., 2016; Tilston-Lunel et al., 2015). Although found to be nonessential for viral growth in some mammalian and insect cell lines, the NSs protein is a type-1 interferon antagonist and has the ability to modulate apoptosis of infected cells, which is part of the host immune response (Blakqori et al., 2007; Eifan et al., 2013; Kohl et al., 2003).

While the NSm protein has been studied less extensively than the NSs protein, it has been shown to be associated with viral infection and replication (Leventhal et al., 2021). The function of the NSm protein differs from that of the NSs protein since it does not impair a virus's ability to infect mammalian cells (Shi et al., 2006). However, experiments have demonstrated that the lack of NSm in BUNV leads to immature viral particle accumulation, signifying a potential role in viral assembly (Fontana et al., 2008). Deletion of NSm in Rift Valley fever virus (RVFV), a phlebovirus, can lead to lower infection and dissemination rates in mosquitoes compared to the wild-type virus (Crabtree et al., 2012). Additionally, AKAV and BUNV without a functional

NSm protein had impaired growth in both mammalian and insect cells (Ishihara et al., 2016; Shi et al., 2006).

Although the single deletion of NSs or NSm can attenuate the virulence phenotype of RVFV, the deletion of both the NSs and NSm proteins has the most significant attenuating effect (Crabtree et al., 2012). Several groups have developed a reverse genetics system to generate a recombinant virus that lacks both the NSs and NSm proteins (Bird et al., 2011; Brennan et al., 2011; Crabtree et al., 2012). This method potentially creates a safe and immunogenic live-attenuated vaccine without the risk of reversion. This technique has also been demonstrated with the deletion of NSs and NSm in SBV, creating promising candidates for the development of safe and effective SBV veterinary vaccines (Kraatz et al., 2015).

Although live-attenuated vaccines are often more efficient in both the onset of immunity and duration of immunity, autogenous inactivated vaccines can be approved by veterinarians when no commercially licensed vaccine is available. Inactivated vaccines are considered safe and useful tools to prevent the spread of emerging diseases. In addition, several inactivated vaccines have been developed against AKAV and SBV with the ability to induce neutralizing antibodies and prevent viremia after a challenge infection (Kim et al., 2011; Wernike et al., 2013).

Previously, Dunlop *et al.* used a reverse genetics system for CVV to produce a recombinant virus that lacks the NSs gene, e.g., rCVVdelNSs (Dunlop et al., 2018). They confirmed that the mutant virus was attenuated across several cell lines and IFN protection assays confirmed the role of the CVV NSs protein as a type-1 interferon antagonist in mammalian cells (Dunlop et al., 2018).To further attenuate rCVVdelNSs, the nucleotides 1039 – 1476 were deleted from the M segment creating 2delCVV. The nucleotide deletions were based

off of previous work and the entire NSm reading frame was deleted (Shi et al., 2016; Tilston-Lunel et al., 2015). The recombinant virus then no longer encoded the NSs and NSm gene.

Immunological evaluation and comparison of different Cache Valley vaccine candidates in sheep

The objective of the first experiment was to evaluate and compare the immunogenicity of a candidate live-attenuated vaccine, 2delCVV and an autogenous binary ethylenimine (BEI) inactivated vaccine for CVV (BEI-CVV). The vaccines were evaluated by quantifying the serum neutralizing activity in sheep. Following the primary immunizations, boosters were used to determine if either vaccine increased the titers of neutralizing antibodies that would likely provide a protective immune response.

Growth characteristics of 2delCVV in mosquitoes

Previously, Seligman and Gould raised concerns regarding the potential for arbovirus live-attenuated vaccines to infect mosquitoes (Seligman & Gould, 2004). Herein, we determine th capacity of the 2delCVV candidate vaccine to replicate in mosquitoes as compared to the CVV 6V633 wild-type strain. The deletion of the virulence factors reduced the ability of RVFV to enter, replicate, and disseminate from the midgut epithelial cells (Kading et al., 2014). In addition, the attenuated phenotype of BUNV NSs deletion mutant has also included the reduced multiplication kinetics in infected mosquitoes (Szemiel et al., 2012).

We have previously proven that *Ae. albopictus* and *Cx. tarsalis* are competent vectors for CVV (Ayers et al., 2019; Ayers et al., 2018). *Ae. albopictus* have a broader geographic distribution throughout North America, therefore they are considered an important vector for the

endemic transmission of CVV and an appropriate model system to study the attenuating effect caused by the simultaneous deletion of NSs and NSm (Dieme et al., 2022; Kamal et al., 2018). In this study, *Ae. albopictus* were intrathoracically inoculated with either the CVV-6V633 wild-type strain or 2delCVV to investigate the replication kinetics. These data from both studies have identified the possibility of a promising vaccine candidate for CVV and potentially other related bunyaviruses.

Materials and methods

Cell lines

African green monkey kidney epithelial Vero76 cells were maintained in Leibovitz's L-15 media (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), 10% tryptose phosphate broth (Sigma-Aldrich, St. Louis, MO, USA), penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA), and L-glutamine (Thermo Fisher Scientific, Waltham, MA, USA) as previously described (Huang et al., 2015). The cells were cultured at 37°C and used for propagation of virus stocks, candidate vaccines, and the processing of experimental samples, as previously described (Huang et al., 2015).

Viruses and candidate vaccines

The prototype 6V633 wild-type strain of CVV, originally isolated from infected *Cs. inornata* in Cache Valley, Utah, in 1956 (Holden & Hess, 1959), was obtained from the collection in the laboratory of Dr. Richard M. Elliot (Watret et al., 1985). Sequences of all three genomic segments have been determined in a previously published study (GenBank accession

numbers: KX100133.1, KX100134.1 and KX100135.1) (Groseth et al., 2017). This wild-type strain was generated by two passages in Vero76 cells and used in all oral challenge experiments for the determination of vector competence

The live-attenuated 2delCVV candidate vaccine was obtained from the collection in the laboratory of Dr. Alain Kohl and created as previously described (Dunlop et al., 2018). To create the 2delCVV candidate vaccine, a reverse genetics system of CVV was used (Dunlop et al., 2018). As previously described, the deletion of the NSs gene was originally achieved by introducing two stop codons in the open reading frame (ORF) encoding the NSs protein (Dunlop et al., 2018). To further attenuate the virulence phenotype of CVV, nucleotides 1039 – 1476 that encode the ORF of the NSm gene were deleted using site-directed mutagenesis. Hence, the resulting recombinant 2delCVV no longer expresses the NSs and NSm genes.

The CVV 6V633 wild-type strain was used for the autogenous BEI-inactivated candidate vaccine (BEI-CVV). The protocol used for inactivating a virus using BEI was previously described with porcine reproductive and respiratory syndrome virus (Bahnemann, 1990). The inactivation of CVV using BEI is described below.

Preparation of BEI was performed by first making 1% beta-naphthol violet in water, as a pH indicator, and adding it to 0.175M NaOH. 2-bromo-ethylamine HBr (BEA) crystals were dissolved into the NaOH solution to produce 0.1M solution of BEA. For the reaction of BEI synthesis, 0.1M BEA in the NaOH was incubated at 37°C for 30 – 60 minutes or until color of the solution changed from purple to orange. The BEI was then stored in a 4°C refrigerator until needed. The next step was to prepare 0.1M sodium thiosulfate in nano pure water. Once combined, the solution was filtered using either a membrane (0.22 µm) or by autoclaving.

For the inactivation of CVV, virus stock was quickly thawed using a 37°C water bath. The 0.1M BEI stock solution was added to the virus to get a final concentration of BEI 1.5 mM. The virus with the BEI solution was then vortexed to completely mix, then incubated at 37°C for 24 hours. After incubation is complete, the BEI was neutralized with 1M sterile sodium thiosulfate, vortexed, and incubated for 2 hours at 37°C. All of the BEI-inactivated CVV was stored in individual 2ml Eppendorf tubes in a -80°C freezer.

The inactivation of the virus was then confirmed with Vero76 cells. A viral infectivity profile was also performed to ensure complete inactivation, using a method similar to plaque assay, as previously described (Baer & Kehn-Hall, 2014; Nuckols et al., 2015). 24-well plates were seeded with Vero76 cells using confluent cell culture flasks and incubated at 37°C for at least five hours to allow the cells to adhere to the bottom of the wells. The BEI inactivated CVV was quickly thawed in a 37°C water bath and then serially diluted 10-fold with L-15 media (10^0 – 10^{-6}), the CVV 6V633 wild-type strain was used as a positive control. Media was removed from each well of the 24-well plate containing the cells and 50 µl of the sample was added per well in duplicates. A set of negative control (L-15 media) and positive control (CVV 6V633 strain) were included in each assay. The inoculum was absorbed at 37°C for 60 minutes with gentle agitation every 15 minutes. After the absorption period, the supernatant was removed, and the wells were rinsed with 1 ml of DPBS/well. The DPBS was removed and 1 ml of 1.5% methyl cellulose overlay was added to each well. The plates were then sealed with parafilm and incubated at 37°C for five days.

The 1.5% methyl cellulose overlay was prepared by mixing 7 to 8 grams of methyl cellulose with 280 ml of molecular biology grade sterile water in a 500 ml round media bottle. With the addition of a magnetic stir bar, the contents were then autoclaved at the liquid setting.

After autoclaving, 50 ml of chilled TPB, 250 ml of chilled 2x L-15 media, 30 ml of FBS, and 10 ml of antibiotics and L-glutamine mixture (100 U/ml penicillin, 100 µg/ml of streptomycin, and 2 mM of L-glutamine) were added to the bottle. The bottle was then spun for at least 10 hours, until the media became uniformly viscous, using the magnetic stirrer. The overlay was then stored in a 4°C refrigerator and tested for sterility at 37°C prior to use.

After five days, the 24-well plates were removed from the incubator and each well was fixed with 1 ml of 10% formalin solution (formaldehyde diluted in PBS) for 30 minutes. The overlay and fixative were then removed, and each well was stained with 1% crystal violet solution (0.5% crystal violet powder and 50% methanol in deionized water). After gently rinsing the plates, the plaques were counted, and the titer of infectious viruses were calculated in plaque forming units (pfu)/ml.

Before immunization, an aluminum (Al)-hydroxide adjuvant was added to the inactivated CVV. Al-hydroxide is a widely used vaccine adjuvant because it efficiently boosts the immune responses against vaccine antigens, therefore, it is present in most ovine commercial vaccines (Burakova et al., 2018). The AI-hydroxide adjuvant used in this experiment was Invivogen 2% aluminum hydroxide gel at a 1:1 ratio with the BEI-CVV.

Animal experiment and design

The following experimental procedures and handling of live animals were approved by the Kansas State University (K-State) Institutional Animal Care and Use Committee (IACUC). All methods were carried out in accordance with the approved protocol and relevant regulations. All animal work was conducted in the Large Animal Research Center (LARC) in biosafety level 2 agriculture (BSL2-Ag) conditions. Animals were allowed an acclimation period of five days in

the BSL2-Ag housing prior to the start of the experiments. Throughout the experiment, all animals were given *ad libitum* access to fresh water and fed a commercial grade pellet ration according to the animal's body weight.

The immunogenicity of a candidate live attenuated vaccine for CVV and an autogenous inactivated vaccine for CVV was evaluated and compared. In addition, booster immunizations were used to determine if either vaccine induce a sufficient amount of neutralizing antibodies that would likely confer protection. Twenty-four six-month-old male Rambouillet lambs were assigned to one of the following vaccine groups: the 2delCVV vaccine group (n = 10), the BEI-CVV group (n = 10), or the L-15 media group (n = 4).

Prior to initial immunization, all animals were determined to be healthy and seronegative to CVV through the analysis of collected serum using PRNT. On day 0 of the experiment, lambs were immunized subcutaneously at their fore flank (right behind their elbow) with their corresponding immunization: 1 ml 2delCVV, 1 ml BEI-CVV with the addition of the aluminum hydroxide adjuvant, or 1 ml of L-15 media. Lambs then received booster immunizations on days 21 and 42 post initial immunization. 4 ml of blood was collected from each animal on days 3, 5, 7, 14, 20, 35, 41, 56, and 63 post initial immunization. Due to frequent sampling, the blood volume collected did not exceed 1% of the total blood n for each animal. Serum samples were then obtained through the centrifugation of the coagulated blood at 2,000 x g for 10 minutes at 4°C and stored in a -80°C freezer for later analysis. The experiment ended 63 days post initial immunization and the sheep were euthanized. For the duration of the study, animals were monitored daily for any clinical signs including fever (>40°C), depression, weight loss, respiratory distress, lameness, neurological signs, and vaccine site reactions. **Figure 3-1** illustrates the timeline of the experiment.

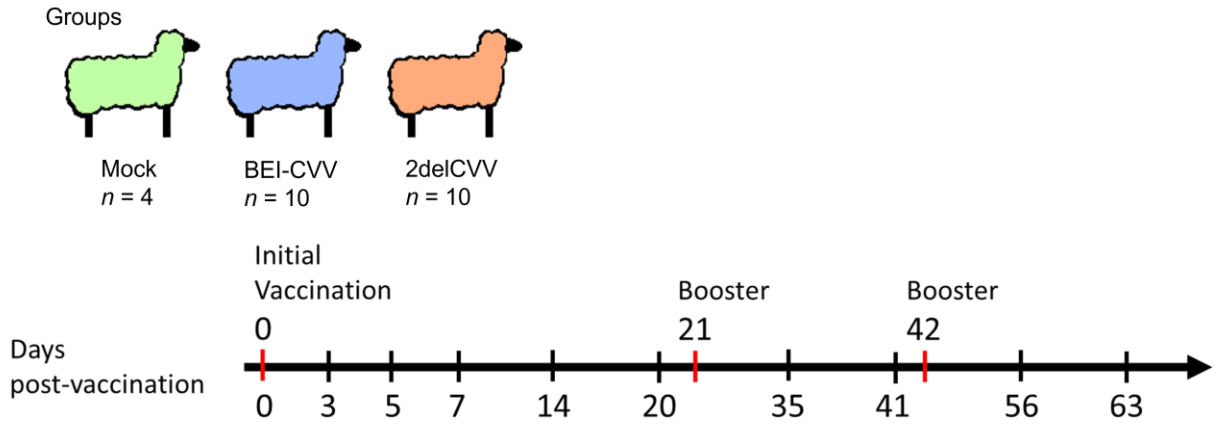

Figure 3-1. Experimental design.

Rambouillet ram lambs (L-15 media, *n* = 4; BEI-CVV, *n* = 10; 2delCVV, *n* = 10) were immunized subcutaneously on day 0 with booster immunizations administered on day 21 and 42 post initial immunization. Serum samples were collected on days 0, 3, 5, 7, 14, 20, 35, 41, 56, and 63 of the study for the assessment of neutralizing antibody activity.

Plaque reduction neutralization test

To determine neutralizing antibody titers, plaque reduction neutralization tests (PRNT) were performed as previously described (Roehrig et al., 2008). 24-well plates were seeded with Vero76 cells using confluent cell culture flasks and incubated at 37°C for at least five hours to allow the cells to adhere to the bottom of the wells. Serum samples were heat inactivated at 56°C for 30 minutes in a water bath to inactivate complement. The samples were then serially diluted 2-fold starting at 1:5 to 1:160 dilutions in 96-well plates. Approximately 30 pfu of CVV 6V633 was added to each serum concentration and incubated for one hour at 37°C. Media was removed from the 24-well plates containing the Vero76 cells and 50 µl of the serum-virus mixture was added into the appropriate wells. A set of virus-only (positive control) and L-15 only (negative control) was included in each assay. The plates were then incubated at 37°C for one hour with

gently agitation every 15 minutes to allow for absorption. After the absorption period, the wells were washed with DPBS and overlaid with 1% methyl cellulose. The plates were sealed with parafilm and incubated at 37°C for five days.

After the five days of incubation, the wells were fixed with 10% formalin solution and then stained with 1% crystal violet stain. Plaques were then counted, and the neutralizing antibody titers were calculated based on 50% or greater reduction in plaque counts ($PRNT_{50}$).

Statistical analysis for neutralizing antibody response in sheep

All statistical analyses were conducted using the GraphPad Prism (version 8.1.2) program (GraphPad Software Inc.). The Shapiro-Wilk test was used to test for normal distribution. If the data does not follow normal distribution, nonparametric tests can be used. $PRNT_{50}$ titers were compared between the two vaccine groups on each day using Mann-Whitney U tests. Kruskal-Wallis tests with post hoc Dunn's multiple pairwise comparison tests were used to compare the $PRNT_{50}$ titers produced on different days in each vaccine group. To evaluate the significant difference between the two vaccine groups over the duration of the study, a two-way ANOVA was performed.

Mosquitoes and intrathoracic inoculation

Inoculation was performed with 7-10-day-old female *Ae. albopictus* (F_4), which were derived from eggs collected from the city of Trenton, Mercer County, NJ, USA, in July 2016. Mosquitoes were reared at 28°C, relative humidity of 80%, and a 12h light:12h dark photoperiod. For intrathoracic inoculation, mosquitoes were cold anesthetized on ice, transferred to a secure glove box, and then inoculated with approximately 0.5ul of either viral stock or the

2delCVV candidate vaccine, as previously described (Huang et al., 2020). Up to three mosquitoes were collected after injection and titrated to confirm the presence of infectious viruses. Intrathoracically inoculated mosquitoes were then collected by mechanical aspiration at 7 days post infection (dpi) for analysis. After collection, whole mosquitoes were frozen in individual 2ml Eppendorf tubes containing 2.8mm metal beads, 96 µl L-15 media, and 4 µl amphotericin.

Detection of infectious viruses in mosquitoes

The overall infection status of each individual mosquito was determined by the detection of CVV in the whole carcasses using the tissue culture infectious dose 50% ($TCID_{50}$)-based titration method with VERO76 cells, as previously described (Ayers et al., 2019; Higgs et al., 2006; Huang et al., 2015). All samples were homogenized using a TissueLyser II apparatus (Qiagen, Germantown, MD) at 26 Hz for four minutes and titrated by $TCID_{50}$ with Vero76 cells (Higgs et al., 2006). Each well of a 96-well plate, except for the first column, received 90 µl of L-15 media. The empty wells were then filled with 100 µl of each mosquito sample, loaded in duplicate, followed by a 10-fold serial dilution. 100 µl of Vero76 cells was then placed in each well. Plates were sealed with parafilm and incubated for 7 days at 37°C. After incubation, individual wells were stained with 200 µl of amido black stain containing 1% amido black B10 suspended in 10% glacial acetic acid and 35% isopropanol aqueous solution at room temperature for 30 minutes. Each plate was then washed with regular tap water and visually evaluated for cytopathic effect. Titers were calculated using the Reed-Muench method (Reed & Muench, 1938). Infectivity of mosquitoes was used to determine the infection rate and multiplication kinetics of the wild-type CVV 6V633 strain and the 2delCVV strain. Infection rates were then

calculated using the percentage of infected mosquitoes among all mosquitoes tested at each time-point. Growth kinetics of CVV in infected mosquitoes was determined based on the titers of CVV in the whole mosquitoes at 7 dpi.

Statistical analysis for replication kinetics

The infection rate percentages were compared using Chi-square test with Yate's correction. Titers of infected mosquitoes were compared with Mann-Whitney rank sum test between the two groups with the infectious titers following non-normal distribution. All statistical analyses were conducted using GraphPad (San Diego, CA, USA) and Excel software (Redmond, WA, USA).

Results

Immunological evaluation and comparison of different CVV vaccine candidates

Prior to immunization, all animals were healthy and seronegative to CVV. Animals from the live-attenuated 2delCVV candidate vaccine group presented no observable adverse clinical signs, nor weight loss or increased rectal temperatures were noted. Sheep from the autogenous BEI-CVV vaccine groups did not display weight loss or increased rectal temperatures however, all animals had swelling and a hard nodule averaging 3 cm in diameter present at the injection site after vaccination that persisted for the duration of the study.

Serum neutralizing antibody titers

Animals were bled on days 3, 5, 7, and 14 to determine immune responses following the primary immunization, and on days 20, 35, 41, 56, and 63 to determine if booster immunizations would increase the immunogenicity and produce a long-lasting neutralizing antibody response

with each candidate vaccine. All animals immunized with the candidate LAV seroconverted above the 1:10 immune protection threshold after a single immunization on day 20 post initial immunization. There was no significant difference in the neutralizing antibodies elicited by both vaccines when compared (**Figure 3-2**). However, on day 63 post initial immunization, the 2delCVV candidate vaccine induced a slightly higher neutralizing antibody response than the autogenous vaccine, although it did not reach statistical significance (**Figure 3-2**). Importantly, immunization of BEI-CVV only led to transient neutralizing antibody responses. The serum $PRNT_{50}$ titers of twenty percent of animals (2/10) receiving BEI-CVV waned to 5 and 10, whereas all animals receiving 2delCVV maintained robust neutralizing activity.

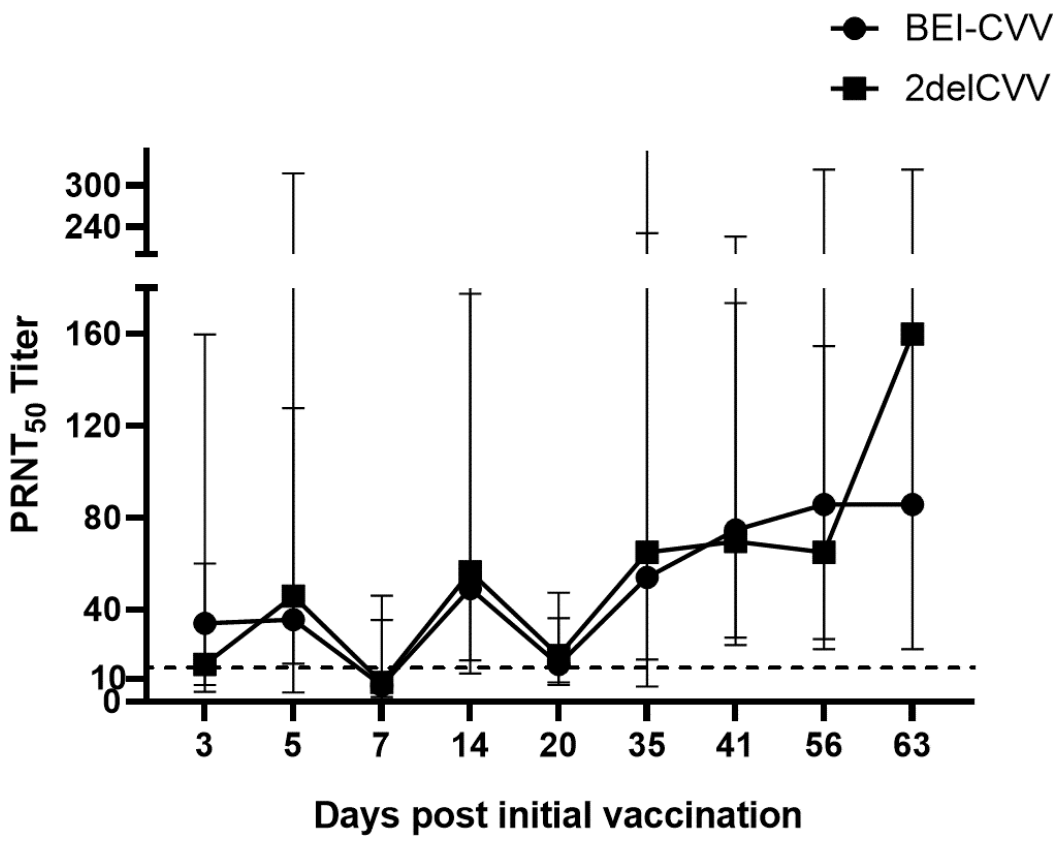

Figure 3-2. Geometric mean $PRNT_{50}$ titer of 2delCVV vaccinated and BEI-CVV vaccinated sheep.

The different candidate vaccines on each day were statistically compared using the Mann-Whitney rank sum test. The data were also compared together using a 2-way ANOVA. Error bars indicate the geometric standard deviation.

Multiplication kinetics of a candidate live-attenuated vaccine for CVV in *Ae. albopictus*

After evaluating the immunogenicity and attenuation of the candidate live-attenuated vaccine, we evaluated the replication kinetics of 2delCVV through intrathoracic inoculation in *Ae. albopictus*. As expected, intrathoracic injection with either the CVV 6V633 strain or the 2delCVV candidate vaccine led to the establishment of infection in *Ae. albopictus*. There was no

significant difference in the infection rates produced by 2delCVV and the CVV 6V633 strain at 7dpi (CVV 6V633: 83.1% [49/59] *vs* 2delCVV: 97.5% [39/40], Chi-square test with Yates's correction: $\chi^2 = 3.682$, $df = 1$, $p = 0.0550$) (**Table 3-1**). Mosquitoes injected with the 2delCVV candidate vaccine had significantly lower infectivity at 7dpi than mosquitoes infected with wtCVV (CVV 6V633: 6.0 $\log_{10}$TCID$_{50}$/ml vs 2delCVV [median titer]: 3.5 $\log_{10}$TCID$_{50}$/ml; Mann-Whitney test: $U = 75.5$, $p < 0.0010$) (**Figure 3-3**). In conclusion, the deletion of NSs and NSm genes significantly reduces the multiplication kinetics of CVV in mosquitoes.

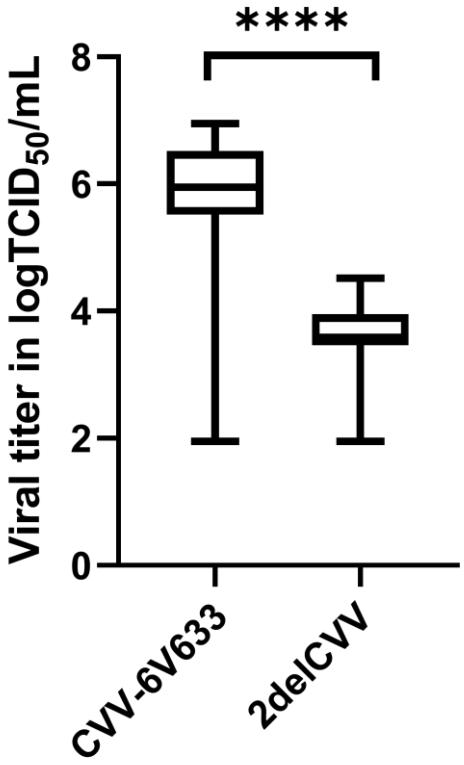

Figure 3-3. Titers in *Aedes albopictus* at 7 dpi.

A Mann-Whitney test was used to compare the viral titers between the two groups of mosquitoes (****, $p < 0.001$). The maximum and minimum values are displayed by the vertical lines connecting the largest and smallest viral titer in the data set. The horizontal bar represents the median titer of whole mosquitoes.

Table 3-1. Comparison of infection rates in *Aedes albopictus* mosquitoes intrathoracically injected with CVV-6V633 or 2delCVV.

Group	Mosquitoes tested	7 dpi
CVV-6V633	59	83.1% (49/59)
2delCVV	40	97.5% (39/40)

Discussion

The creation of a safe and immunogenic vaccine for emerging bunyaviruses without the ability to revert to virulence has become a priority. It is also important that the vaccine is unable to grow in mosquitoes, preventing mosquitoes from transmitting the virulent vaccine to livestock or humans. To prevent this, a reverse genetics system was developed to modify the segmented viruses' genome segments (Dunlop et al., 2018). The method of deleting the NSs and the NSm proteins has previously been successful with other related viruses. For example, the Bunyamwera Orthobunyavirus, lacking the NSs protein, was unable to replicate in *Ae. aegypti* and *Ae. albopictus* cell lines (Szemiel et al., 2012). Therefore, an attenuated virus lacking the NSs protein would likely reduce or prevent replication in mosquitoes.

The deletion of NSm greatly reduced the infection, dissemination, and transmission rates of RVFV in *Ae. aegypti* (Kading et al., 2014). Additionally, the simultaneous deletion of NSs and NSm from the RVFV genome resulted in the highest level of attenuation of virus replication and failed to infect *Ae. aegypti* mosquitoes (Crabtree et al., 2012). Data generated in this study demonstrated that the 2delCVV vaccine candidate was restricted in its replication in *Ae.*

albopictus compared to the CVV 6V633 wild-type strain. These data suggest the NSs and NSm proteins are necessary for efficient growth in *Aedes* mosquitoes.

Although reproductive losses are the most significant economic burden to the sheep industry, there are no vaccines available for CVV. In this study, we evaluated the immunogenicity of a candidate 2delCVV live-attenuated vaccine and an autogenous vaccine for CVV. While both vaccines provided a neutralizing antibody response past the threshold of immune protection, animals immunized with the 2delCVV candidate vaccine developed higher serum neutralizing titers at 63 days post immunization. We conclude that 2delCVV is superior in immunogenicity and can be further evaluated as a candidate veterinary LAV.

Inactivated vaccines generally have a high safety profile and can be developed within a relatively short period of time making them an ideal method for producing vaccines. The inactivated candidate vaccine used in this study was created similarly to one of the inactivated vaccine candidates used against SBV (Wernike et al., 2013). They were both inactivated with binary ethylenimine with the addition of an aluminum hydroxide adjuvant. The outcome was also similar with most of the animals developing detectable neutralizing antibodies upon vaccination. However, as expected, the autogenous vaccine seemed to produce a significantly higher neutralizing antibody response after the first and second booster immunization. This indicates that the first dose may only give partial protection and the maximum immune response is not achieved until after one or two booster immunizations. Since there was no significant difference in the neutralizing antibody titers produced by the first and second booster, only a single booster may be necessary for the BEI-CVV candidate to induce a long-lasting neutralizing antibody response.

A challenge study would be necessary to determine actual protection in pregnant sheep against the fetal malformations and abortions that infection with CVV causes. Such challenges could not be performed during the current studies. Since CVV does not normally display clinical symptoms in adult ruminants, challenging the sheep used in this study would not have given relevant information on protection. The future challenge study conducted will need to test for safety and protection in pregnant ruminants. Previously an inactivated vaccine for RVFV reduced viremia with a lack of clinical signs in a vaccinated lamb, however, there were no detectable neutralizing antibodies (Kortekaas et al., 2012). This could potentially mean that the inactivated vaccine could provide adequate protection with only one single immunization without the need for booster immunizations. In addition, the use of adjuvants is sometimes problematic. Aluminum hydroxide adjuvants have been shown to cause granulomas and potentially act as a contributor to severe wasting syndrome (de Miguel et al., 2021; Echeverria et al., 2020).

Live-attenuated vaccines are typically considered to be more efficacious than autogenous inactivated vaccines. Modified live vaccines were initially based on random introduced mutations via serial passages in cell culture or in the presence of chemical mutagens, however there have been incidences where the vaccine will revert to a wild-type or pathogenic form (Henderson, 2005; Shams, 2005; Vannie et al., 2007; Weyer et al., 2016). Reassortment between orthobunyaviruses has also been shown to be a major component in orthobunyavirus evolution (Briese et al., 2007). Another limitation is the inability for these vaccines to be differentiation injected from vaccinated animals (DIVA) vaccines. The live-attenuated candidate 2delCVV vaccine was created with the inability of the virus to express the NSs and NSm genes. This attenuated the virus so it would elicit an immune response without the ability to revert to full

virulence. The complete deletion of genes could possibly enable us to distinguish vaccinated from field-infected animals. This could potentially be achieved through the detection of the wild-type virus using an NSs or NSm antibody and through the detection of the mutant virus using an anti-NSs or anti-NSm antibody as previously described (Bird et al., 2011) A CVV double deletion mutant virus would also have significant advantages similar to the double deletion mutant for SBV, including its ability to be transmitted by insect vectors (Kraatz et al., 2015).

While experiments with BUNV have demonstrated how NSs was nonessential in mosquito cell lines, NSs was shown to be important for the infection of mosquitoes (Szemiel et al., 2012). Additionally, NSm is important for the virulence in insect hosts (Kraatz et al., 2015). Demonstrating the need for both NSs and NSm for efficient growth of the virus in arthropod cells and suggesting that spread into vector populations is unlikely for 2delCVV.

As expected, the autogenous BEI-CVV vaccine was capable of eliciting a neutralizing antibody response, however, a booster immunization would be needed and there was an adverse reaction at the injection site. The 2delCVV candidate vaccine not only produced a neutralizing antibody response through the duration of the study that could confer protection, but it also produced a more robust neutralizing antibody response at the end of the study when compared to the autogenous vaccine. Therefore, through the deletion of the NSs and NSm genes, an immunogenic vaccine for CVV was developed. While protection studies are warranted in sheep, the attenuated phenotype and immunogenicity of 2delCVV in sheep and the reduced replication kinetics observed in mosquitoes provides a basis for the further development of immunogenic vaccines for CVV and other related orthobunyaviruses.

Chapter 4 - Evaluating the neutralizing antibody response produced by a candidate live-attenuated Rift Valley fever virus vaccine with a two-segmented genome using the MP12 strain

The experiment detailed in this chapter demonstrates the immunogenicity of a candidate live-attenuated RVFV vaccine lacking the NSs and NSm genes (r2segMP12) in CD-1 mice. The work in this chapter has been submitted for peer review and publication in *Viral Immunology*.

Introduction

Rift Valley fever virus (RVFV; Phenuiviridae, Phlebovirus) is a clinically important mosquito-borne pathogen causing disease in both humans and ruminants. Although most humans have no clinical signs, others develop flu-like symptoms with headaches, fever, or myalgia (Hartman, 2017; Laughlin et al., 1979; Wichgers Schreur et al., 2020). In addition, a number of infections can progress to severe diseases including encephalitis, hemorrhagic fever, or thrombosis, which can result in death (Ikegami & Makino, 2011). While humans are considered dead-end hosts for RVFV, ruminants, especially sheep and goats, act as amplifying hosts (Chevalier et al., 2010; Hartman, 2017). In livestock, death from the disease is most commonly caused by abortion storms with abortion rates of up to 100% (Chevalier et al., 2010; Hartman, 2017; Ikegami & Makino, 2011; Laughlin et al., 1979; Wichgers Schreur et al., 2014; Wichgers Schreur et al., 2021).

Vaccination is the most effective method of preventing and controlling RVFV outbreaks (Ikegami & Makino, 2009). Currently, there are no licensed vaccines or antiviral treatments for humans or animals in non-endemic countries (Faburay et al., 2017). Multiple veterinary vaccines

are available and commonly used in livestock in endemic counties, including the Smithburn strain, Clone 13 strain, and the conditionally licensed MP12 strain (Alhaj, 2016). Although the Smithburn vaccine is immunogenic, it cannot be used for the immunization of young and pregnant ruminants because it retains its ability to cause stillbirths, neonatal death, and abortions in sheep (Botros et al., 2006; Coetzer & Barnard, 1977; Ikegami & Makino, 2009; Kamal, 2009). The Clone 13 strain vaccine has been shown to be safe and effective in lambs, cattle, and pregnant ewes, but is also partially attenuated, as observed with vertical transmission and teratogenic effects in ewes after the administration of high doses (Dungu et al., 2010; B. Makoschey et al., 2016).

Several approaches have been taken to address the limitations of the currently available candidate RVFV vaccines through the gene-deletions of one or both virulence factors (NSs and NSm) (Billecocq et al., 2008; Dunlop et al., 2018; Habjan et al., 2008; Ikegami et al., 2006). NSs is a non-structural protein that facilitates evasion of the host innate immune system, while the NSm non-structural protein promotes suppression of apoptosis in infected hosts (Brennan et al., 2014; Ikegami & Makino, 2011). More importantly, the deletion of NSm has previously resulted in the reduced ability of RVFV to infect, replicate, and disseminate from the midgut epithelial cells in *Aedes* mosquitoes (Kading et al., 2014).

Brennan et.al constructed a candidate vaccine based on the attenuated MP12 strain that lacks the NSs and NSm genes in a reconfigured two-segmented genome, designated r2segMP12 (Brennan et al., 2011). The r2segMP12 is similar to the three-segmented RVFV vaccine candidate and was rationally designed based off of previously published work (Bird, Albarino, et al., 2008; Ikegami et al., 2006; Won et al., 2006; S. Won et al., 2007). Both the three-segmented RVFV vaccine and the r2segMP12 candidate lack the NSs and NSm genes, however, the

r2segMP12 candidate uses the MP12 parental strain as the backbone, not the ZH501 virulent RVFV strain.

In this study, the immunogenicity of the r2segMP12 vaccine candidate was evaluated by quantifying the serum neutralizing activity in CD-1 mice. Groups of mice were subcutaneously inoculated with different titers of the vaccine candidate on day 0, followed by a booster dose on day 21 post initial immunization. Serum samples were collected on days 20 and 42 post initial immunization and evaluated using plaque reduction neutralization tests for a neutralizing antibody titer at or above the threshold antibody level for protection.

The neutralizing antibody response produced by the different titers of r2segMP12 were compared between the use of a single dose versus a single dose followed by a booster dose. Neutralizing antibodies produced following the r2segMP12 vaccine and the MP12 parental strain vaccine were also examined. Altogether, these data provide important information regarding the efficacy of the r2segMP12 vaccine, and whether or not attenuated vaccine candidates with both the NSs and NSm genes deleted still elicit a robust immunogenic response.

Materials and methods

Cell lines

African green monkey kidney epithelial Vero76 cells were maintained in Leibovitz's L-15 media (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), 10% tryptose phosphate broth (Sigma-Aldrich, St. Louis, MO, USA), penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA), and L-glutamine (Thermo Fisher Scientific, Waltham, MA, USA) as previously described (Huang et al., 2015). The cells were cultured at 37°C and used for propagation of virus stocks,

the candidate vaccine, and the processing of experimental samples, as previously described (Huang et al., 2015).

Viruses

The recombinant vaccine candidate (r2segMP12) for RVFV was produced in a previously published study (Brennan et al., 2011). The recombinant MP12 strain containing a two-segmented genome, lacking the NSs and NSm genes, was titered in African green monkey kidney epithelial (Vero76) cells maintained in Leibovitz's L-15 media (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum, 10% tryptose phosphate broth, penicillin/streptomycin, and L-glutamine. The rMP12 parental strain vaccine was generated using a control rescue experiment as previously described (Brennan et al., 2011). It was then titered in Vero76 cells as a positive control (Caplen et al., 1985).

Animal experiment and design

The following experimental procedures and handling of live animals were approved by the K-State Institutional Animal Care and Use Committee (IACUC). All methods were carried out in accordance with the approved protocol and relevant regulations. All animal work was conducted in the animal biosafety level-2 (ABSL-2) laboratories in the K-State College of Veterinary Medicine.

The immunogenicity of a recombinant RVFV-vaccine, containing a bi-segmented viral genome was evaluated in outbred CD-1 mice. To determine the immunization regimens required to elicit protective neutralizing antibody responses, fifty 3–4-week-old, outbred CD-1 mice (Charles River, Raleigh NC) were subcutaneously immunized. Animals were placed into groups

of five representing regimens using up to 10^5 pfu of infectious viruses in a single immunization or two immunizations (**Table 4-1**). Prior to initial immunization, all animals were determined to be healthy and seronegative to RVFV through the analysis of collected serum using PRNT.

Table 4-1. Immunization regimen of CD-1 mice (Dosages are calculated in pfu/mouse).

Group	Number of mice	Dosage of 1st immunization (Day 0)	Dosage of 2nd immunization (Day 21)
1	5	10^3 (r2segMP12*)	no injection
2	5	10^4 (r2segMP12*)	no injection
3	5	10^5 (r2segMP12*)	no injection
4	5	0 (sterile media; mock control)	no injection
5	5	10^5 (parental strain MP-12 control)	no injection
6	5	10^3 (r2segMP12*)	10^5 (r2segMP12*)
7	5	10^4 (r2segMP12*)	10^5 (r2segMP12*)
8	5	10^5 (r2segMP12*)	10^5 (r2segMP12*)
9	5	sterile media; mock control	sterile media; mock control
10	5	10^5 (parental MP-12 strain control)	10^5 (parental MP-12 strain control)

* r2segMP12 represents the recombinant vaccine candidate for RVFV.

All mice received an initial immunization on day 0 of the experiment. The regimen of a single immunization administered at an increasing dosage per group (n = 5) was included to determine the amount of neutralizing antibodies produced by the r2segMP12 strain. Four groups of mice (n = 5) received a second immunization at 10^5 PFU on day 21 of the experiment, to evaluate the need for a booster immunization. In addition to the experimental groups that received the r2segMP12 vaccine candidate, four additional groups (n = 5) were designated as control groups, with two positive control groups receiving the MP12 vaccine at 10^5 PFU and two negative control groups receiving an equal volume of sterile culture L-15 media.

All animals were maintained for 6-weeks after the initial immunization. For the duration of the study, animals were monitored daily for any observable adverse clinical signs. Mice were immobilized using an isoflurane vaporizer prior to blood collection. 0.1ml of whole blood samples were collected from the lateral saphenous vein from immunized animals on day 20 post-

initial immunization using a 22g needle. Serum samples were obtained through centrifugation of coagulated blood at 2,000 x g for 10 minutes at 4°C and used for the detection of neutralizing antibodies using $PRNT_{50}$. At 42 days post initial immunization, a terminal bleed was performed by cardiac puncture following isoflurane anesthesia and death was confirmed by cervical dislocation. **Figure 4-1** illustrates the timeline and experimental design of the study.

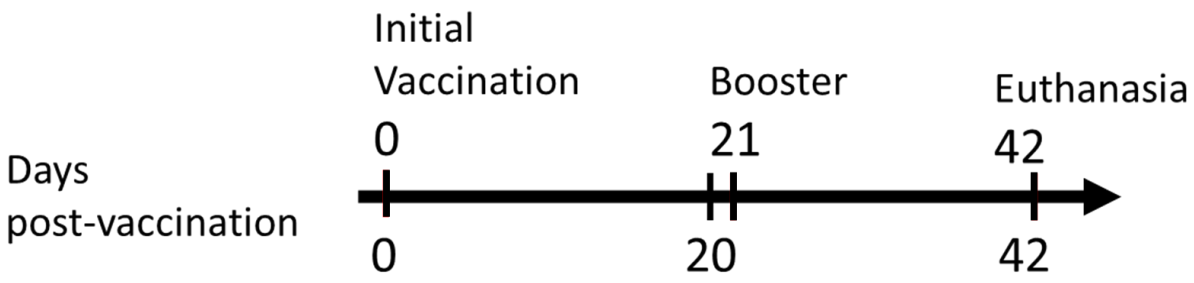

Figure 4-1. Timeline of experimental mouse design.
Mice were immunized subcutaneously on day 0 with a booster immunization administered on day 21 post initial immunization to groups receiving a booster. Serum samples were collected on days 0, 20, and 42 of the study for the assessment of neutralizing antibody activity.

Plaque reduction neutralization test

As performed in the previous experiments, neutralizing antibody titers were determined using PRNT assays (Roehrig et al., 2008). 24-well plates were seeded with Vero76 cells using confluent cell culture flasks and incubated at 37°C for at least five hours to allow the cells to adhere to the bottom of the wells. Serum samples were heat inactivated at 56°C for 30 minutes in a water bath to inactivate complement. The samples were then serially diluted 2-fold starting at 1:10 to 1:320 dilutions in 96-well plates. Approximately 50 pfu of the MP12 vaccine strain was added to each serum concentration and incubated for one hour at 37°C. Media was removed from

the 24-well plates containing the Vero76 cells and 50 µl of the serum-virus mixture was added into the appropriate wells. A set of virus-only (positive control) and L-15 only (negative control) was included in each assay. The plates were then incubated at 37°C for one hour with gently agitation every 15 minutes to allow for absorption. After the absorption period, the wells were washed with DPBS and overlaid with 1% methyl cellulose. The plates were sealed with parafilm and incubated at 37°C for five days.

After the five days of incubation, the wells were fixed with 10% formalin solution and then stained with 1% crystal violet stain. Plaques were then counted, and the neutralizing antibody titers were calculated based on 50% or greater reduction in plaque counts. Seroconversion was defined using the cut-off of 1:10 $PRNT_{50}$ titer, a seropositive threshold commonly used for assessing the neutralizing antibody responses elicited by arbovirus vaccines (Julander et al., 2011; Roehrig et al., 2008; Van Gessel et al., 2011).

Statistical analysis

The $PRNT_{50}$ titers of animals receiving each dosage of the vaccine candidate were compared on day 20 using a Kruskal-Wallis test followed by Dunnett's test as the post hoc multiple comparison procedure, including a comparison of each dosage to the parental strain MP12 positive control. Using a Kruskal-Wallis test followed by a Dunnett's test post hoc, $PRNT_{50}$ titers of animals receiving a single dose of the r2segMP12 vaccine candidate at varying titers were compared to mice receiving both an initial dose of the r2segMP12 vaccine candidate at varying titers and a booster vaccine at 10^5 PFU at 42 days post immunization. Lastly, $PRNT_{50}$ titers of animals receiving only a single dosage of the r2segMP12 vaccine candidate at 10^5 PFU were compared to animals receiving the MP12 vaccine at 10^5 PFU at 42 days post immunization

with a Mann-Whitney U test. All tests were performed using the GraphPad Prism (version 8.1.2) program (GraphPad Software Inc.).

Results

Serum neutralizing antibody titers

Animals from all groups did not show any observable adverse clinical signs during the experiment. All animals were bled at 20 dpi to evaluate if one single immunization with the r2segMP12 vaccine candidate would produce a neutralizing antibody response above the protective threshold (1:10). All but one mouse immunized with the r2segMP12 vaccine candidate seroconverted above the 1:10 dilution threshold after a single immunization (**Figure 4-2**). Therefore, the r2segMP12 vaccine candidate was capable to elicit neutralizing antibody responses in CD-1 mice at dosages between 10^3 and 10^5 PFU. Mice which received a single immunization of the r2segMP12 vaccine candidate at 10^5 PFU produced a significantly higher number of neutralizing antibodies than mice that received a single immunization of the r2segMP12 vaccine candidate at 10^3 PFU (**Figure 4-2**, $p = 0.0139$), demonstrating a dose response relationship in the vaccine immunogenicity. Importantly, the comparison of immunogenicity with the MP12 vaccine strain suggests the superior immunogenicity of the r2segMP12 strain. Mice immunized with r2segMP12 at a titer of 10^5 PFU had a significantly higher neutralizing antibody response compared to mice that received the MP12 vaccine at the same titer (**Figure 4-2**, $p = 0.0079$).

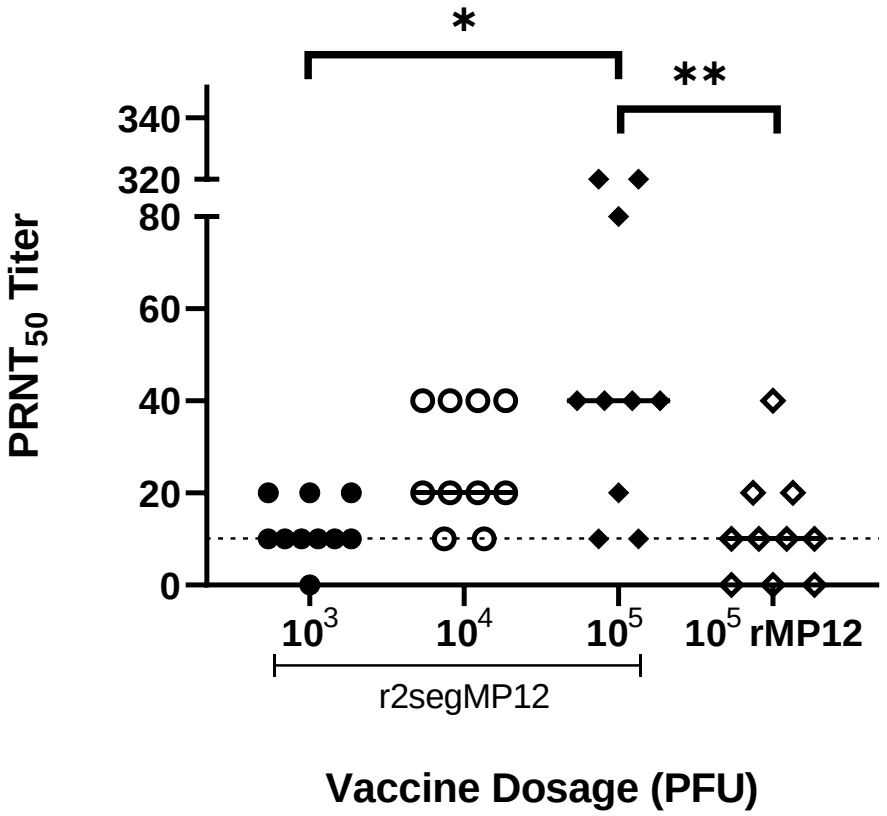

Figure 4-2. Comparison of r2segMP12 and MP12 neutralizing antibody response.

CD-1 mice (*n* = 10 per group) were administered either r2segMP12 stain at one of three titers (10^3, 10^4, 10^5 PFU) or MP12 strain (10^5 PFU). Serum was collected at 20 dpi and antibody titer was measured by PRNT50 with a 1:10 neutralizing antibody titer used as the threshold for the correlate of protection (dotted line). Using the Kruskal-Wallis test with Dunnett's post-hoc multiple comparison test. The bar lines represent the medians of the values from that group of animals. PFU=plaque forming units.

To determine if a booster immunization of the r2segMP12 strain can increase immunogenicity and produce long-lasting neutralizing antibody responses, the $PRNT_{50}$ titers was measured in mice that received varying initial titers of the r2segMP12, followed by a booster at 21 dpi. Animals in groups 6, 7, and 8 (**Table 4-1**) received a booster of the r2segMP12 vaccine candidate at a titer of 10^5 PFU at 21 dpi (**Figure 4-3**). No significant differences in the $PRNT_{50}$

titers between mice that received one single immunization of the r2segMP12 strain and mice that received a final boost at 21 dpi. Additionally, two mice that received the r2segMP12 vaccine at 10^4 PFU with the addition of the booster had a slightly higher neutralizing antibody response than mice that received the 10^5 PFU vaccine and booster, there was no significant difference in the group as a whole. These results demonstrated the immunogenicity of the r2segMP12 strain, with neutralizing antibody titers suggestive of protection.

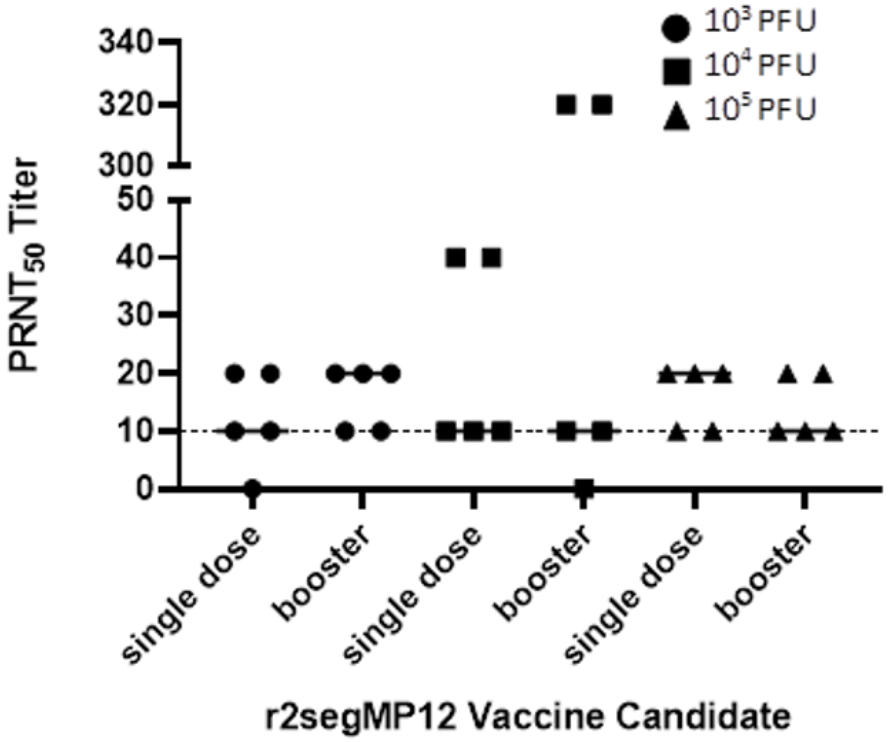

Figure 4-3. Comparison of $PRNT_{50}$ titers from different doses of the r2segMP12 vaccine candidate on 42dpi.

CD-1 mice (n = 5 per group) were administered either a single dose of r2segMP12 (at either 10^3 (filled circles), 10^4 (filled squares), or 10^5 (filled triangles) PFU), or two doses (single dose and a booster dose) at the same titer (primary immunization at either 10^3, 10^4, or 10^5 PFU; booster at 10^5 PFU). Serum neutralizing activity was measured by $PRNT_{50}$. Using a Kruskal-Wallis test followed by a Dunnett's test as the post hoc multiple comparison procedure. The bars represent the median and the threshold of protection is marked by the dotted line.

Given the observation that a single dose of the r2segMP12 vaccine candidate at 10^5 PFU elicited serum neutralizing antibody response at 42 dpi, the level of antibody production was next compared with the neutralizing antibody response induced by a single dose of the MP12 vaccine administered at the same titer. Intriguingly, a single dose of the r2segMP12 strain produced a significantly higher titer of neutralizing antibodies than the MP12 vaccine (**Figure 4-4**, *, p = 0.0238). In addition, four out of five mice lost all neutralizing antibodies at the end of

the study, demonstrating the need for a booster immunization. These data suggest that the r2segMP12 strain is superior to the MP12 vaccine in eliciting neutralizing antibody responses in mice.

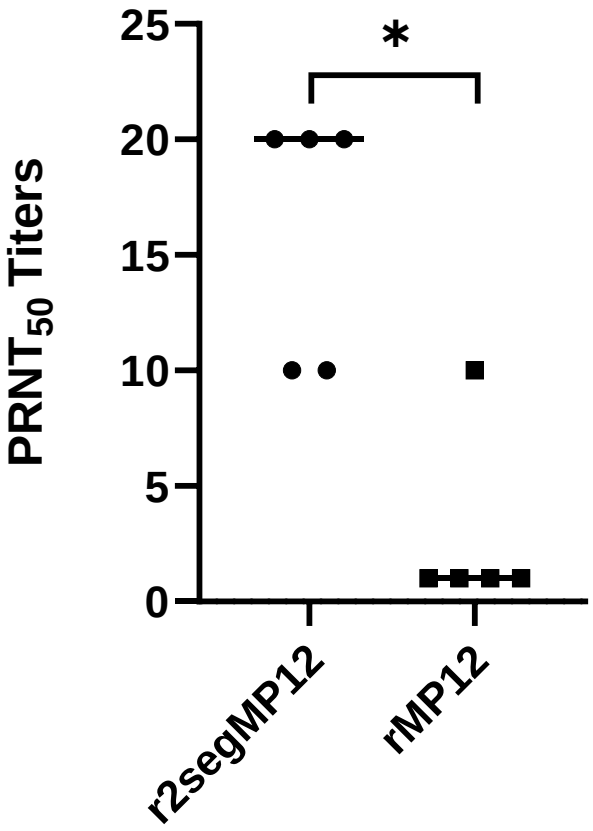

Figure 4-4. Comparison of $PRNT_{50}$ titers from a single immunization of vaccines at the same titer 42 dpi.

CD-1 mice (n = 5 per group) were administered a single dose of either r2segMP12 or MP12 at 10^5 PFU and antibody titer was measured by $PRNT_{50}$. A Mann-Whitney test was used to compare the r2segMP12 candidate and the MP12 vaccine neutralizing antibody responses (*, p = 0.0238). The bars represent the median.

We conclude that the superior immunogenicity of the r2segMP12 strain warrants its advancement in the process of vaccine development, including challenge protection studies conducted in sheep. Collectively, these data suggest that the r2segMP12 strain is immunogenic and can elicit neutralizing antibody responses in CD-1 mice that received one single immunization.

Discussion

Due to the impact of RVFV on both human and livestock health, there is an ongoing effort to prevent and control RVFV; however, the limitations of each vaccine, multiple doses required, and expenses to maintain these regimens has made it difficult (Hunter et al., 2002; Mackenzie, 1935; Pittman et al., 1999; Randall et al., 1962). Live-attenuated vaccines have been developed for RVFV in an effort to eliminate the need for booster inoculations, but these vaccines have demonstrated that in certain instances they can retain virulence and cause teratogenic effects and abortions in livestock (Hunter et al., 2002; Morrill et al., 1997). These limitations cause safety concerns, especially in nonendemic areas during epidemic periods.

There is an urgent need to develop a new vaccine against RVFV. Therefore, this study sought to establish the immunogenicity of a recombinant RVFV-vaccine, containing a two-segmented viral genome in outbred CD-1 mice. Altogether, the observations made demonstrated that a single dose of the r2segMP12 strain induced a neutralizing antibody response in mice. The neutralizing antibody responses were suggestive of protection because they presented the 1:10 PRNT50 titer which represents the threshold of protection upon challenge with RVFV. Although previous studies have shown certain recombinant vaccines to be protective against lethal RVFV strains with a neutralizing antibody titer as low as 1:4 (Wallace et al., 2006).

The candidate vaccine used in this study was rationally designed through the deletion of the virulence factors, NSs and NSm, which is based on previous work (Bird et al., 2011; Ikegami et al., 2006; Sungyong Won et al., 2007). Previously, the generation of viruses lacking the NSs gene established the product is not essential for replication in mice, making NSs an accessory protein (Anne Bridgen et al., 2001). However, there is now evidence of the NSs protein contributing to RVFV disease outcome in mice by modulating host cell features and defense mechanisms (Léger et al., 2020). In comparison, the Clone 13 vaccine demonstrated to be avirulent in mice and highly immunogenic (Muller et al., 1995). However, even with the deletion of the NSs segment in the Clone 13 strain, it has been reported to cause stillbirths and fetal infections when administered in an overdose to pregnant ewes in their first trimester (Birgit Makoschey et al., 2016).

While other RVFV candidate live-attenuated vaccines have been developed through the deliberate deletion of NSs and NSm genes and demonstrated to be safe and immunogenic in mice and pregnant sheep (Bird, Albarino, et al., 2008; Bird et al., 2011), our work has important implications for the development of RVFV candidate live-attenuated vaccines. Although these vaccines were made using similar methods, the r2segMP12 strain with a two-segmented genome will have a reduced likelihood for the reversion to the virulence phenotype. Additionally, the r2segMP12 strain proves that the double deletion of NSs and NSm genes does not cause reduced immunogenicity of the MP12 strain (Brennan et al., 2011).

The results of this study also determined that the r2segMP12 strain elicited a significantly higher level of neutralizing antibody response than the conditionally licensed MP12 vaccine at 20 and 42 dpi. In addition, the r2segMP12 strain does not express the NSs and NSm proteins, providing the basis for differentiating infected from vaccinated animals (DIVA). Currently, there

is little DIVA capacity amongst most available candidate RVFV vaccines contain the whole genome. However, r2segMP12 was designed to be easily DIVA detectable via qPCR.

We conclude that the superior immunogenicity of the r2segMP12 strain warrants its advancement in the process of vaccine development, including challenge protection studies conducted in sheep, which are the amplifying hosts for RVFV. Future experiments will focus on the characterization of the immune response induced by r2segMP12 and its ability to protect against a lethal RVFV challenge.

Chapter 5 - Discussion and future directions

The work presented in this dissertation provides information to further understand the transmission of CVV in North America and discusses the potential development of live-attenuated candidate vaccines for emerging bunyaviruses. In this chapter the major conclusions of this work are summarized and discussed, and future directions to continue to improve bunyavirus prevention, control, and mitigation strategies are provided.

Vector competence of North American mosquitoes to bunyaviruses

Knowledge regarding the relative importance of competent mosquito vector species is critical not only for our understanding of transmission cycles of arboviruses but also for the development of vector control strategies for bunyaviruses. A good example for why vector competence studies are important was determining the role of *Ae. albopictus* in transmitting WNV in Louisiana, where the *Culex quinquefasciatus* population was targeted and successfully suppressed by vector control (Palmisano et al., 2005; Vanlandingham et al., 2016). In this dissertation, determining vector competence was important to assess whether medically important North American mosquito species were capable of transmitting orthobunyaviruses, i.e., CVV. Identification of new mosquito species capable of transmitting CVV would advance our knowledge of where CVV outbreaks may occur, what species are likely responsible for transmission of CVV from animals to humans, and the development of effective mitigation strategies. For example, a previous study modelled RVFV in the state of Kansas due to the presence of potentially competent RVFV mosquito vectors and potentially susceptible cattle available in high numbers. They concluded that there is a general lack of mosquito control in Kansas which increases the risk of RVFV transmission, should it be introduced (Scoglio et al.,

2016). Successful mosquito management requires intervening at some point during the mosquito's life cycle; therefore, mosquito control should be a major priority after determining competent vectors to target.

As the world continues to evolve with climate change, increased global travel, and more importation and exportation of livestock, the risk of emerging and re-emerging pathogens has increased. Especially since higher temperatures can cause an increase in growth rates of vector populations, decrease the time between blood meals, shorten the extrinsic incubation time, accelerate the virus evolution rate, and increase viral transmission efficiency to birds (Kilpatrick et al., 2008; Paz et al., 2013; Paz & Semenza, 2013; Reisen, Fang, & Martinez, 2006; Ruiz et al., 2010). The emerging pathogen chosen for its evaluation in North American mosquitoes in this dissertation was CVV. Cache Valley virus was selected because of its inconsistency in livestock outbreaks and rare but severe disease it causes in humans with no explanation as to why or how. In addition, CVV can serve as a surrogate for viruses such as La Crosse orthobunyavirus or Rift Valley fever phlebovirus. Understanding the spread and transmission of CVV through previously unidentified competent mosquito vectors could assist in furthering our knowledge of the spread and transmission of other bunyaviruses.

One North American *Culex* species and two invasive *Aedes* species mosquitoes, now established in North America, were competent vectors for CVV based on laboratory infection, disseminated infection, and CVV transmission in the saliva, demonstrating their ability to potentially transmit CVV. Cache Valley virus was recently detected for the first time in *Ae. albopictus* collected in New York, further proposing the potential involvement of this species in the transmission cycle (Dieme et al., 2022). This discovery suggests that this species may potentially be continuing to expand its range in the northeastern United States. The current

geographic distribution of *Ae. albopictus* mosquitoes is shown in **Figure 5-1** , which shows that *Aedes* species mosquitoes are not likely found in New York (Centers for Disease Control and Prevention, 2020a). The distribution and spread of this species is coincidental with the high prevalence of CVV in white-tailed deer in New York (Dupuis et al., 2020).

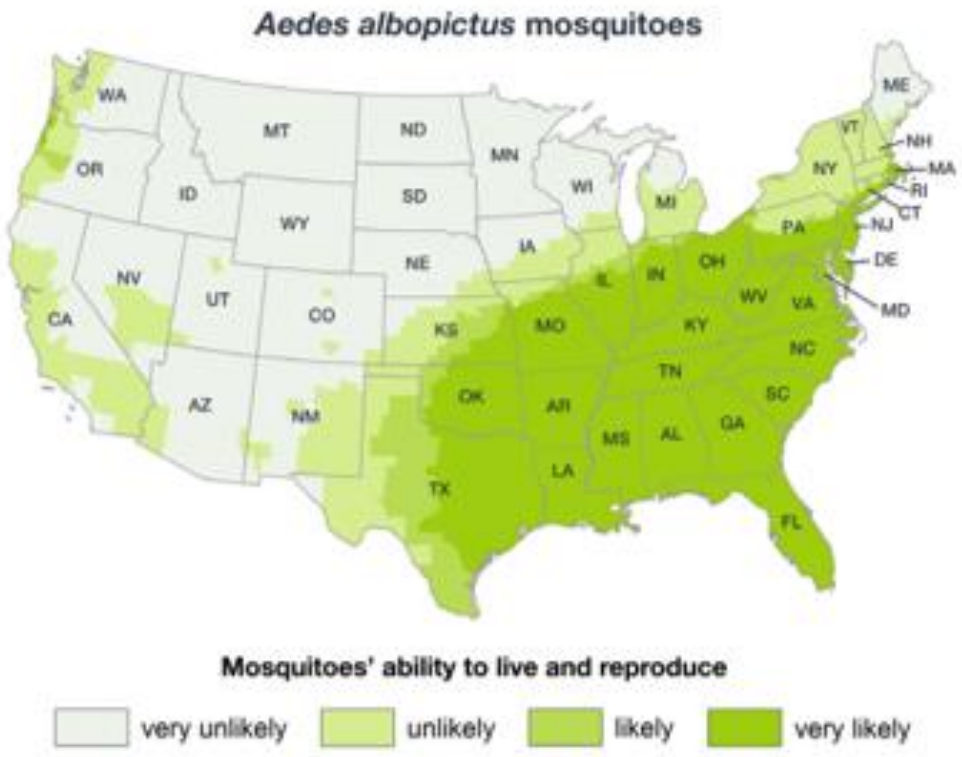

Figure 5-1. Potential range of *Aedes albopictus* mosquitoes.

These maps represent CDC's best estimate of the potential range of *Ae. albopictus* in the United States. Maps do not represent risk for spread of disease. Image modified from (Centers for Disease Control and Prevention, 2020a); Published in the public domain.

Mosquito control for the specific species evaluated in this dissertation is not only important for controlling CVV but also for RVFV, if an introduction were to occur. Several studies have documented vector competence for RVFV in European and North American *Aedes* and *Culex* species mosquitoes, increasing the possibility of viral spread beyond its current distribution following a natural introduction or an intentional release event (Chevalier et al., 2010; Turell, Wilson, et al., 2010). *Ae. aegypti*, *Ae. albopictus*, and *Cx. tarsalis* were previously tested for vector competence for circulating strains of RVFV in Africa. All three species were competent vectors for RVFV based on the laboratory infection, dissemination infection, and detection in the saliva (Iranpour et al., 2011; McIntosh et al., 1980; Turell et al., 1988; Turell, Wilson, et al., 2010). In addition, RVFV was isolated from *Ae. aegypti* during one of the outbreaks in Sudan (Seufi & Galal, 2010). This suggests that targeting these species for surveillance and control would be beneficial to improve the efficiency of bunyavirus prevention programs, as previously described for flaviviruses (Huang et al., 2015).

Development of bunyavirus vaccines

Vaccination programs are the most effective method for preventing and controlling outbreaks (Ikegami & Makino, 2009). This has partially been proven when a massive outbreak of Bluetongue virus (BTV) in 2006 was controlled by animal movement restrictions and intensive vaccination (Maclachlan & Mayo, 2013). More than 100 million animals were vaccinated throughout Europe which rapidly decreased BTV infections (Zientara et al., 2010).

Part of this dissertation work investigated candidate live-attenuated vaccines for two emerging bunyaviruses, CVV and RVFV. However, working with highly pathogenic vaccines can sometimes be difficult; this is because biosafety level (BSL) – 3 or even – 4 containment

laboratories are necessary for various studies. Cache Valley virus is a BSL-2 agent whereas RVFV is a BSL-3 select agent. Both need a vaccine for the prevention and control of future outbreaks but, allowing us to work with a BSL-2 agent initially prepared us for the hazardous work required when studying RVFV.

The candidate vaccines used in this dissertation were based off of previous work that studied the genomic segments using reverse genetics to create effective vaccines (Bird, Albarino, et al., 2008; Brennan et al., 2011; Dunlop et al., 2018; Ikegami et al., 2006; Won et al., 2006; S. Won et al., 2007). The first study was determining the neutralizing antibody response of a candidate vaccine with a live-attenuated CVV vaccine lacking the NSs and NSm genes called 2delCVV. The neutralizing antibody response induced by 2delCVV was then compared to an autogenous BEI-inactivated vaccine for CVV. This study was carried out similar to a previous study done where the neutralizing antibody response produced by a glycoprotein subunit vaccine was evaluated in sheep without a challenge experiment (Faburay et al., 2014). Although, this is the first CVV vaccine to be developed, vaccines for other orthobunyaviruses, such as SBV, have determined that the deletion of both NSs and NSm are necessary for protection (Kraatz et al., 2015).

While a challenge experiment was not conducted, the study provides initial data determining the attenuation and immunogenicity of a CVV vaccine virus lacking the virulence genes. A previous study with RVFV in humans and nonhuman primates showed that a $PRNT_{80}$ titer of ≥ 40 is protective against virulent RVFV (Ikegami, 2017). Although this needs more investigation, the 2delCVV candidate vaccine could potentially confer protection after the initial and booster vaccine. This is suggested because the neutralizing antibodies produced by 2delCVV were higher than the 1:40 $PRNT_{50}$ titer. However, protection by neutralizing antibodies to JEV is

well established that such neutralizing antibody titers of ≥ 1:10 are accepted as evidence of protection and seroconversion (Fujisaki, 1975; Tsai, 1990; Turtle et al., 2016). These results also suggests that through the deletion of the NSs and NSm genes, the virus is attenuated and immunogenic.

The neutralizing antibody response by the autogenous BEI-inactivated vaccine was also similar to the 2delCVV candidate however, there was a reaction at the injection site. Whilst inactivated vaccines are considered safe and effective in preventing clinical disease, the reaction at the injection site may not be ideal for those who use ruminants as show animals. This reaction could potentially be from the aluminum hydroxide adjuvant which has previously caused inflammation at the site of the injection that sometimes corresponded to granulomas (Asin et al., 2019; He et al., 2015). Aluminum hydroxide may also contribute to severe wasting syndrome which can cause anorexia and progressive weight loss (Asin et al., 2021; de Miguel et al., 2021).

The attenuation of the 2delCVV candidate vaccine was also evaluated in competent mosquito vectors. Data generated in this study demonstrated that the 2delCVV vaccine candidate was restricted in its replication in intrathoracically inoculated *Ae. albopictus* compared to the CVV 6V633 wild-type strain. Along with the reduced multiplication kinetics of the 2delCVV strain in the vertebrate host SFT-R cells, this candidate CVV LAV has a lower potential of transmission by mosquitoes, similar to the attenuated phenotype of the YF 17D vaccine strain in *Ae. aegypti* (Danet et al., 2019; Dunlop et al., 2018; McElroy et al., 2006). Although the YF 17D vaccine could infect mosquitoes, it was unable to disseminate to the secondary tissues and failed to transmit to a novel host (Danet et al., 2019). This suggests that the midgut escape barrier and the midgut infection barrier restrict this live-attenuated vaccine from replication and transmission. Similarly, *Ae. aegypti* and *Ae. albopictus* were orally challenged with ChimeriVax

vaccine candidates and resulted in such low titers that the ability of these mosquitoes to facilitate transmission would be unlikely (Higgs et al., 2006). Whilst restriction of 2delCVV was observed in this study, the genetic mechanisms as to why these vaccine viruses do not replicate and disseminate in mosquitoes is still unknown.

Findings in this study also suggest the NSs and NSm genes are necessary for efficient growth in *Aedes* species mosquitoes. The specific role of NSs and NSm in mosquitoes still needs to be defined since NSs is not essential for viral growth in cell culture and NSm has been said to be dispensable for virus replication in mosquito cell lines (Elliott, 2014; Tilston-Lunel et al., 2015). Although unnecessary for viral growth in cell lines, the presence of these genes seem to be necessary to overcome the cellular defenses in the midgut. In a previous study, the deletion of NSs in BUNV was unable to bypass the cellular defenses however, when these barriers were overcome, the vaccine viruses was capable of spreading to the secondary tissues and salivary glands (Szemiel et al., 2012). More recently, a human vaccine candidate for RVFV lacking the NSm gene was unable to infect, replicate or be transmitted by multiple mosquito species (Campbell et al., 2021). Therefore, the NSs and NSm genes seem to be necessary for the efficient replication and transmission of bunyaviruses in mosquitoes.

Lastly, the immunogenicity of a candidate live-attenuated vaccine for RVFV lacking the NSs and NSm genes (r2segMP12), similar to the CVV candidate vaccine, was evaluated in CD-1 mice. Although there are a variety of candidate vaccines for RVFV under development, the major difference in the one used in this dissertation is the r2segMP12 candidate vaccine has a two-segmented genome instead of a three segmented genome. Based off this major difference in the genome compared to the wild-type strain, there is potential in this vaccine being DIVA compatible. However, further studies will need to be conducted.

Future directions

The work in this dissertation investigated bunyavirus control, prevention, and mitigation strategies. This included the determination of competent vectors in North America for CVV and assessing two different live-attenuated candidate vaccines for CVV and RVFV. Two *Aedes* species and one *Culex* species found in North America were identified as being susceptible to infection with CVV and capable of transmission. These findings suggest the importance of surveillance and control strategies to monitor emerging bunyaviruses and prevent their introduction. This could include more educational resources for CVV to prevent outbreaks and expand diagnostic capacity to detect CVV infections by immunoassays. This could also include increased and better diagnostic testing for CVV in humans since most cases are under-reported or misdiagnosed. Having increased surveillance of CVV frequency in the vector and monitoring the evolution of vector populations could be a way to predict future viral outbreaks. Additionally, testing herd immunity against CVV could be another suitable approach. Controlling insect populations have been described as another prophylactic measure to decrease virus transmission and prevent infection (Claine et al., 2015).

Vaccination is another preventative measure that would reduce the impact of CVV infection, control the spread of RVFV in endemic areas, prevent the introduction of RVFV into new areas, and reduce the possibility of outbreaks of emerging bunyaviruses. While the live-attenuated candidate vaccine for CVV produced a robust neutralizing antibody response in sheep, future work will need to evaluate the vaccines conferred protection against CVV challenge infection in pregnant sheep.

Previously, the reassortment between two related recombinant bunyaviruses, CVV and Kairi virus (KRIV) was evaluated in the T7 RNA polymerase-expressing cell line BSR-T7/5. Future studies should determine which related orthobunyaviruses share an overlapping geographic distribution. Subsequently, the recombinant form of the viruses identified should be evaluated for environmental reassortment risk that could be associated with vaccination.

The 2delCVV candidate vaccine was also intrathoracically injected into a susceptible vector for CVV. However, future studies should examine the ability of *Ae. albopictus* to transmit this candidate vaccine. Additionally, if the major vector for CVV is identified, the infection, dissemination, and transmission rate of 2delCVV should be determined.

Lastly, the most immunogenic dosage of the RVFV candidate vaccine, r2segMP12 was determined in mice and compared to the parental RVFV MP12 strain. To advance the development of r2segMP12, the immunogenicity of the vaccine should be evaluated in the amplification host, sheep. Additionally, the capability of r2segMP12 to induce a long-lasting immune response without a booster immunization should be determined. Future experiments will focus on the characterization of the immune response induced by r2segMP12 and its ability to protect against a lethal RVFV challenge.

Findings from our studies provide a better understanding of which species could be responsible for the enzootic spillover of CVV from viremic animals to humans. Since there are no licensed vaccines for use in humans, the improvement of prevention and control strategies is necessary. The work provided in this dissertation also provides insight into the immunogenicity of two candidate live-attenuate vaccines for bunyaviruses. However, it is important both candidate vaccines are optimized, and future vaccine safety and efficacy studies are conducted.

The methods for developing these two candidate vaccines could potentially lead to the development of a platform vaccine for other related bunyaviruses.

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