Exploring the Ecology of Orthobunyaviruses in Virginia and their Pathogenesis in Murine and Poultry Models

Krisangel López

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Entomology

Albert J. Auguste, Chair

Sally L. Paulson

Luis E. Escobar

Tamaki Yuri

Gillian Eastwood

December 16th, 2024

Blacksburg, VA

Keywords: animal models, arbovirus, Cache Valley virus, mosquitoes, surveillance

Exploring the Ecology of Orthobunyaviruses in Virginia and their Pathogenesis in Murine and Poultry Models

Krisangel López

ABSTRACT

Cache Valley virus (CVV) is a vector-borne, negative-sense RNA virus, in the genus *orthobunyavirus*. Cache Valley Virus is a widespread pathogen in North America, and since its first isolation in 1956, has been associated with multiple epizootics of CVV in ruminants, leading to spontaneous abortions and congenital malformations. As such, CVV is a virus of high economic relevance, but little is known about fundamental aspects of its biology. To address this gap of knowledge, I conducted a series of studies to better understand the pathogenesis and ecology of CVV. This work is divided into two facets; the first is the development of animal models to assess the pathogenesis of CVV in various host species, and the second is vector surveillance to better understand the ecology of orthobunyaviruses within the Commonwealth of Virginia. In the first two chapters, I address the lack of small animal models to study CVV. First, I developed a murine model and an *in utero* model that mimic the natural progression of disease observed in CVV infection. In the second chapter, I study the growth kinetics of CVV in avian cell lines and in commercial poultry species. In the last chapter, I explore the distribution and diversity of mosquitoes and arthropod-borne viruses in Virginia. Overall, these studies provide insight into CVV pathogenesis and *in utero* transmission, the role of domestic poultry in the maintenance and amplification of CVV, and lastly, evidence of mosquito species range expansion, and high viral diversity across the Commonwealth of Virginia.

Exploring the Ecology of Orthobunyaviruses in Virginia and their Pathogenesis in Murine and Poultry Models

Krisangel López

GENERAL AUDIENCE ABSTRACT

The world's deadliest animal is the mosquito. Mosquitoes can transmit a wide variety of diseases, including viruses. Cache Valley virus is a widespread virus in North America that can be transmitted by different species of mosquitoes. Cache Valley virus is associated with miscarriages and deformities in livestock, particularly in sheep, brain swelling, and death in humans. Nevertheless, studying Cache Valley virus to date has been very difficult as sheep and goats are the only available animals that show signs of illness, and they require large spaces to be studied. My research focused on developing a mouse and a bird infection model that could be used to observe how the virus grows, how the disease develops, and how it is passed down from parent to offspring. I also wanted to assess where Cache Valley virus circulates in nature in the Commonwealth of Virginia. To study Cache Valley virus circulation, mosquitoes were caught at multiple locations using Virginia as a study site and tested for the presence of virus. Although we did not detect Cache Valley virus in these samples, we found Jamestown Canyon virus, another important virus that has previously not been known to occur in Virginia. These studies allowed an in-depth look at Cache Valley virus ecology and shed light on the need for future mosquito monitoring across Commonwealth of Virginia.

Acknowledgements

I would like to thank my advisor Dr. A. Jonathan Auguste for being a great mentor and leader through the years. I feel extremely lucky to be a part of his lab, and of his academic legacy. Thank you to my committee members, Dr. Sally Paulson, Dr. Luis E. Escobar, Dr. Tamaki Yuri, and Dr. Gillian Eastwood for their guidance and invaluable feedback throughout the years. Thank you to all of my collaborators, Dr. Christine Carrington, Dr. Jesse Erasmus, Dr. Chloe Lehondère, Dr. Joanna M. Reinhold, Dr. Orchid Allicock, and Dr. Lin Kang, and also the volunteers that made so much of this work possible. Thank you to all my amazing lab members, Manette Tanelus, Sarah N. Wilson, Dawn I. Auguste, William B. Stone, and Danielle L. Porier, whom all worked diligently by my side, and went above and beyond to help me with assays, fieldwork, and fun hiking trips and themed dinner nights to help keep our spirits up. Thank you to all of my friends and family, both near and far, that have believed in me and supported me for so many years. A huge thank you to my uncle, Dr. Ramon A. Taveras, for always being my biggest inspiration and nurturing both my creativity and love for science from an early age. Lastly, I would like to thank and dedicate this work is dedicated to three of the most important people in my life. First my husband, Dr. John A. Muller, thank you for your love and support, and everything you do for me and our family. Thank you for believing in me as a person and a scientist, and for all your hard work and the dedication you put into this research, which included data collection, processing, analysis and editing. This work could not have been completed without you. Second, to my son Quintin E. Muller, thank you, for teaching me that I am more resilient than I ever imagined, for your unconditional love, and for making every day so much brighter. Finally, my mother, who has sacrificed so much of her life and happiness so that I could pursue my dreams of becoming a scientist. This research is a testament to all the hard work you have put into raising Derek and me

all by yourself. Thank you, mom, for everything you have done for me, this work, is proof that your sacrifices have not been in vain.

Table of Contents

List of Figures

Table of Tables

General Introduction

Throughout the recorded history of communicable disease, viruses have been and continue to be one of the leading causes of morbidity and mortality (Nathanson, 2016). Over time, viral diseases have accounted for millions of deaths per year, with an even higher disease burden in developing countries (Nathanson, 2016). Viruses were first described in 1892 by Dmitri Ivanovski while investigating a disease that was affecting Tabacco plants (Lechevalier, 1972). Ivanovsky deemed the disease a "filterable virus" in reference to its Latin meaning "poison" (Lechevalier, 1972). Although the first virus was not seen until 1935 with the invention of the electron microscope, many viruses were still studied and described via their effects on hosts and immune systems (Mueller & Rouse, 2009). Viruses are now recognized as the most abundant biological entity on planet earth (Koonin & Yutin, 2019). As technology and science has advanced, we have jumped "leaps and bounds" in terms of our understanding of viruses and their true impact, not only in human populations but in most living organisms on this planet.

Viruses

Viruses are microbes made up of nucleic acid segments of either DNA or RNA, encapsulated in protein coats (Louten, 2016). Viruses come in many shapes and sizes and infect theoretically every organism on planet earth (Louten, 2016; Payne, 2017a). Evolutionarily, viruses are a hijacking machine, as they package their genetic material and use the cells of the host they infect to undergo replication (Louten, 2016; Payne, 2017a, 2017b, 2017c). The genetic material in viruses, varies significantly, and can be broken up into two main categories, DNA viruses and RNA viruses (Louten, 2016; Payne, 2017b, 2017c). Within these two categories there are seven subclasses, separated based on type of genetic material and replication strategy of the viruses (Louten, 2016).

The Baltimore classification of viruses goes as follows: double-stranded DNA viruses (Group I), single-stranded DNA viruses (Group II), double-stranded RNA viruses (Group III), positive single-stranded RNA viruses (Group IV), negative single-stranded RNA viruses (Group V), RNA retroviruses (Group VI), and DNA Retroviruses (Group VII) (Koonin & Yutin, 2019; Louten, 2016).

DNA viruses tend to be relatively large, ranging from ~1-2 megabase pairs for the viruses that affect amoebas and algae to ~5-8 kilobase pairs for papillomaviruses (Fleischmann, 1996; Koonin & Yutin, 2019; Szpara & Van Doorslaer, 2021). DNA viruses have significantly more stable genetic structures, and evolutionarily mutate at a much slower rate than RNA viruses (Fleischmann, 1996). This is because DNA viruses have proof-reading mechanisms, which significantly reduces the error rate in their genomes during replication (Fleischmann, 1996; Payne, 2017b). Multiple well-known DNA viruses cause severe disease in humans, including herpesviruses, poxviruses, and papillomaviruses (Payne, 2017b). The transmission of DNA viruses is usually restricted to some form of contact from person to person via touch, sexual encounter, or bodily fluids, among others (Bushman, McCormick, & Sherrill-Mix, 2019). Although rare, there are some interesting transmission outliers. Ranavirus is one of the few known mosquito-associated DNA viruses (Lesbarrères et al., 2012). Ranaviruses cause severe epizootics in amphibians, turtles, and in some fish species. The primary route of transmission for most Ranaviruses is through contaminated water, physical contact, and ingestion of infected tissues (Lesbarrères et al., 2012). Transmission of DNA viruses via mosquitoes is incredibly rare, it is, however, far more common in RNA viruses.

RNA viruses are significantly smaller than their DNA counterparts, ranging in size from 9 kb to 29 kb (Belshaw, Pybus, & Rambaut, 2007), and have very mutation prone genomes because

they lack a proof-reading mechanism in their replication enzymes (Fleischmann, 1996). RNA viruses are divided into various groups. Two of the most important groups of viruses as it pertains to insect-vector transmission are the positive strand RNA viruses (+ssRNA) and the negative strand RNA viruses (-ssRNA). Positive strand RNA viruses have genomes with functioning messenger RNA (mRNA), meaning that upon cell entry they are able to start translating into proteins to develop progeny virions (Louten, 2016; Payne, 2017c). Unlike +ssRNA viruses, -ssRNA, upon entering the cell cannot immediately start replicating, they must first be translated into mRNA to initiate replication (Louten, 2016; Payne, 2017c).

There are many RNA viruses associated with animal and human infections. Examples include, Influenza "Flu" (*Orthomyxoviradae*), SARS-CoV-2 (*Coronaviridae*), Hepatitis (*Hepeviridae*), Human Immunodeficiency virus (H.I.V.) [*Retroviridae*], and, Rabies (*Rhabdoviridae*), just to name a few (Payne, 2017c). Viruses like Influenza and SARS-CoV-2 are responsible for large quotas of the annual morbidity and mortality annually for communicable diseases. SARS-CoV-2 accounts for 8.8 million deaths in 2021 alone (W.H.O., 2024). Viruses like SAR-CoV-2 and Influenza can be transmitted from one host to another without requiring a vector as part of their transmission cycle (Gillim-Ross & Subbarao, 2006). This mode of transmission makes the development of preventative technologies, like vaccines are effective tools toward reducing the burden of disease (Amanna & Slifka, 2020; Gillim-Ross & Subbarao, 2006). Current technologies allow for the development of effective annual vaccines for the Flu and SARS-CoV-2, which are very mutation prone viruses and constantly evolve (Amanna & Slifka, 2020; Gillim-Ross & Subbarao, 2006). There is, however, a large portion of RNA viruses with a much more complicated ecology for which vaccines and anti-virals have been more elusive, the arthropod-borne viruses (arboviruses).

Arboviruses

Arboviruses are viruses that are maintained in nature through transmission cycle that involves a host and a hematophagous insect vector (e.g., mosquitoes, ticks, sandflies, and flies) (Durden & Mullen, 2019). Some of the most recognizable arboviruses, include dengue viruses (DENV), West Nile virus (WNV), Zika virus (ZIKV*)*, chikungunya virus (CHIKV), and Eastern Equine Encephalitis virus (EEEV) (Griffin & Weaver, 2020; Pierson, Lazear, & Diamond, 2020), all of which are transmitted by a mosquito vector. One of the most fascinating aspects of arboviruses is their complicated ecology and their transmission. There are two main modes of arboviral transmission, horizontal and vertical transmission. Simply put, horizontal transmission is the transference of virus between insect vector and host, while vertical transmission is from the adult insect vector to its offspring (Foster & Walker, 2019). The complex ecology of arboviruses makes them incredibly difficult to eradicate, and the evolutionary drivers of arboviruses (e.g., high mutation rate, broad host and vector range, recombination and reassortment) mean they are constantly, adapting to preventative treatments (Foster & Walker, 2019). A great example of this is CHIKV, which in 2005 underwent changes due to evolutionary pressure that made it compatible with a new vector, *Aedes albopictus* mosquitoes. Evolutionary changes in CHIKV (Tsetsarkin et al., 2009), enabled the virus to spread rapidly across novel areas of the world, ultimately leading to the 2013-2014 CHIKV outbreak across the Americas (Tsetsarkin, Vanlandingham, McGee, & Higgs, 2007).

Although arboviruses can be transmitted by a wide variety of arthropod groups, mosquitoes account for the highest global consequence and disease burden. Mosquitoes are considered the deadliest animal in the world, as they are responsible for approximately one million deaths per year (Qureshi, 2018). Mosquitoes vector a wide range of arboviruses, many of significant

consequence to human health (Madewell, 2020). Of over 500 recognized arboviruses, approximately 150 are associated with human disease (Madewell, 2020). In the last 100 years, we have observed a significant increase in arbovirus prevalence (Madewell, 2020). This spike in arbovirus-related disease has amalgamated from a variety of sources, which include, changes in climate change (Robert, Stewart-Ibarra, & Estallo, 2020), ease of travel (e.g., boat, airplane, train, shipping containers) (Escobar, Qiao, & Peterson, 2016), encroachment of human habitation into previously undeveloped land, and habitat fragmentation (da Silva Pessoa Vieira et al., 2022).

Arboviruses with the largest global impact and highest annual disease prevalence include dengue (DENV) with 96 million cases, CHIKV with 693,000 cases, ZIKV with 500,000 cases, yellow fever virus (YFV) with 130,000 cases, Japanese encephalitis virus (JEV) with 42,500 cases, and WNV with 2588 cases (Madewell, 2020). Additionally, we have seen an increase in some re-emerging arboviruses, like Rift Valley virus, Mayaro virus, and La Crosse virus among many others (Madewell, 2020). A lot of these viruses are associated with two "nuisance mosquitoes" *Aedes aegypti* and *Aedes albopictus*, both invasive species to the Americas known to be anthropophilic feeders (Bursali & Simsek, 2024; Sullivan, Gould, & Maneechai, 1971). It is estimated that the economic burden between 1975 and 2020, for *A. aegypti* and *A. albopictus*-related illness alone, was nearly 94.7 billion dollars, a number which is considered a severe underestimation and continuing to rise as does the disease burden (Roiz et al., 2024). The estimates in the above study only took into consideration two species of disease carrying mosquitoes (Roiz et al., 2024), which begs the question: what is the true burden of disease and economic loss due to all mosquito-borne illness? With a significant increase in arboviral disease prevalence globally and the introduction of viruses into naive regions of the world, we need to make the case to further our knowledge in animal model development and arboviruses surveillance worldwide.

There are three key genera of viruses primarily associated with arboviral diseases, Alphaviruses, Orthoflaviviruses, and Orthobunyaviruses (B. Miller, 2008; Barr et al., 2020). Alphaviruses are found globally, with 31 virus species that can be broken up into two groups of pathogenic viruses, the Old World and New World viruses (Griffin & Weaver, 2020). The Old World viruses are usually characterized by acute febrile illness presenting with rash and arthritis (i.e., CHKV), whereas the New World viruses are associated with encephalitis (i.e., EEEV) (Griffin & Weaver, 2020). Orthoflaviviruses are likely the most well-known group of arboviruses, with 54 viral species distributed throughout the world (Pierson et al., 2020). Orthoflaviviruses are found in six out of the seven continents and are responsible for yearly endemic and epidemic outbreaks (Pierson et al., 2020). Orthoflaviviruses cluster based on their ecology of transmission and their natural host, into four groups; the mosquito-borne flaviviruses (*Aedes* vectored or *Culex* vectored), the tick-borne flaviviruses, no known vector flaviviruses, and the insect-specific flaviviruses (Pierson et al., 2020). Some of the most notable and devastating viruses in this group known for high annual morbidity include DENV, ZIKV, YFV, WNV, and JEV (Madewell, 2020). Although, Alphaviruses and Orthoflaviviruses are among the most important genera of my work is focused on Orthobunyaviruses, which are the largest genera of RNA viruses yet continue to be severely understudied (Hartman & Myler, 2023).

Orthobunyaviruses

Bunyavirales is the largest order of viruses and are composed of 12 families (*Arenaviradae*, *Cruliviridae*, *Fimoviridae*, *Hantaviridae*, *Leishbuviridae*, *Mypoviridae*, *Nairoviridae*, *Peribunyaviridae*, *Phasmavirisae*, *Phenuiviridae*, *Tospoviridae*, and *Wupedeviridae*) (Barr, Weber, & Schmaljohn, 2020). Viruses of this order are widely distributed and infect a broad range

of hosts (vertebrates, arthropods, plants, protozoans), with over 200 recognized viral species, many of which are mosquito-borne (Barr et al., 2020; Spiropoulou & Bente, 2020) Most of the viral families within the order are composed of two or three negative sense RNA genome segments (Barr et al., 2020). Out of the 12 families, the largest is *Peribunyaviridae*, with 89 confirmed species, 82 of which belong to the genus *Orthobunyavirus*, which is the only genus in this family associated with human and animal disease (Barr et al., 2020).

Orthobunyavirus virions are pleomorphic and on average measure 108 nm in diameter (Barr et al., 2020; Elliott, 2014). They are composed of three single-stranded negative sense RNA segments. The L segment, which codes for the RNA-dependent RNA polymerase, that is responsible for viral replication and synthesis of the mRNA. The M segment encodes for the surface glycoproteins, Gn and Gc, and nonstructural protein NSm, which are integral for viral entry. Lastly, the S segment, encodes for the nucleocapsid and the nonstructural protein NSs (Barr et al., 2020; Elliott, 2014) (Figure 1a). In addition to their rapid mutation rate, the structure of their tri-segmented genome makes orthobunyaviruses very likely to reassort. Viral reassortment takes place when the same cell is infected with two similar bunyaviruses, and their genetic segments reassort creating new viral strains (Barr et al., 2020; S. McDonald, Nelson, Turner, & Patton, 2016) (Figure1b).

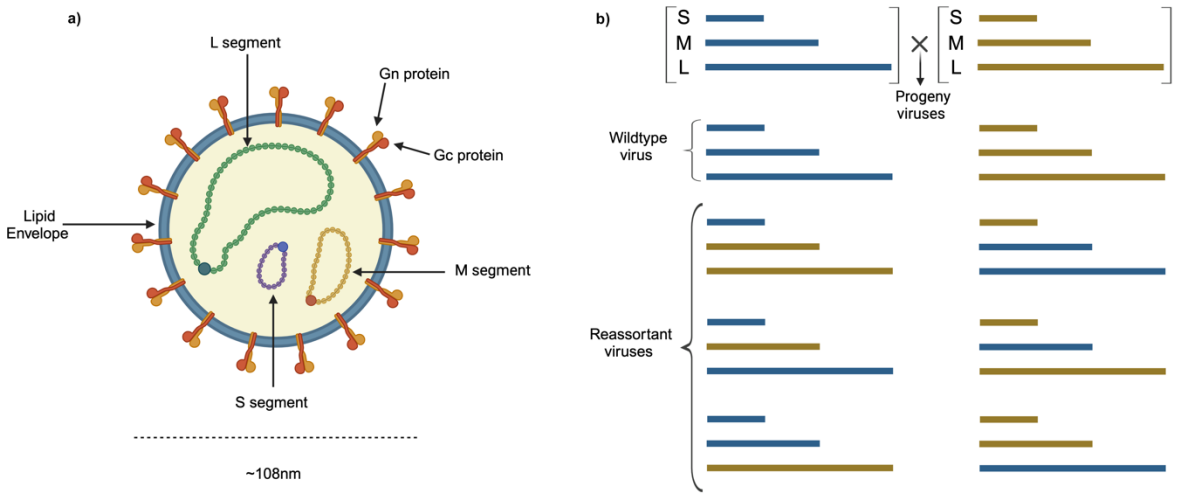


Figure 1. (a) *Orthobunyavirus* virion morphology. The virions are composed of three single-stranded negative sense RNA segments; the L segment, encodes for the RNA-dependent RNA polymerase, responsible for viral replication and synthesis of the mRNA. The M segment encodes for the surface glycoproteins, Gn and Gc, and nonstructural protein NSm, responsible for viral entry. *The S segment* encodes for the nucleocapsid and the nonstructural protein NSs. **(b)** Reassortment of Orthobunyaviruses genome segments. When a cell is coinfected with two genetically similar virus strains, various genotypes can be presented in the progeny virus. Two will be the parental genotype and the remainder with two segments from one parent virus and one from the other parent virus. This figure was adapted from (Barr et al., 2020) and created using biorender.com

Orthobunyaviruses are broken up into 20 serogroups, which are based on their serologic similarity of their complement fixing antibodies, and their hemagglutinating and neutralizing antibodies (Barr et al., 2020; Spiropoulou & Bente, 2020). Out of the 20 serogroups, the most important based on disease outcomes are the California, Bunyamwera and the Simbu serogroups (Barr et al., 2020; Spiropoulou & Bente, 2020). Three of the most important Orthobunyaviruses as it relates to human and wildlife health in the USA are La Crosse virus (LACV) and Jamestown Canyon virus (JCV), both in the California serogroup, and Cache Valley virus (CVV), which is a part of the Bunyamwera serogroup (Barr et al., 2020; Spiropoulou & Bente, 2020).

La Crosse virus is the leading cause of pediatric encephalitis in North America (Harding, Greig, Mascarenhas, Young, & Waddell, 2019). Discovered in 1960 in La Crosse, Wisconsin, it is one of the most important arboviral pathogens in North America with an incidence rate of up to

1.6 cases annually per 100,000 people, and up to 2.27 per 100,000 people under the age of 19 (C.D.C., 2024; Harding et al., 2019). Symptomatic cases of LACV present with febrile illness lasting around three days, with symptoms including nausea, vomiting, and fatigue (C.D.C., 2024; Harding et al., 2019). Neuroinvasive cases of LACV result in encephalitis, long term neurological sequelae and, in rare cases, death (C.D.C., 2024; Harding et al., 2019). Neuro-invasive cases are typically observed in children under the age of 16 (Harding et al., 2019). Historically LACV cases have occurred in Midwestern (e.g., Ohio, Wisconsin, Minnesota, Indiana, Illinois, Iowa), and Appalachian states (e.g., North Carolina, Tennessee, West Virginia, Georgia, Virginia, and Kentucky) (Harding et al., 2019). LACV is primarily transmitted by *Aedes triseriatus* mosquitoes via horizontal and vertical transmission (B. Miller, DeFoliart, & Yuill, 1977). Additionally, recent laboratory studies have demonstrated transmission competence for *Aedes albopictus* and *A. japonicus*, both invasive mosquito species with expanding habitat ranges in the United States (US) (Harding et al., 2019; Rochlin, Ninivaggi, Hutchinson, & Farajollahi, 2013). The primary mammal host of LACV are eastern chipmunks (*Tamias striatus*) and eastern gray squirrels (*Sciurus carolinensis*), whose ranges overlap throughout most of the eastern US and Canada (Harding et al., 2019). As of now, there are no approved interventions such as vaccines or antivirals for LACV which exacerbates its importance (Harding et al., 2019).

Jamestown Canyon virus (JCV) is an emerging arbovirus in North America. Like LACV, JCV is part of the California serogroup of viruses (Evans, Winkler, & Peterson, 2019) and is known to cause acute illness in humans, including febrile encephalitis, meningitis, and meningoencephalitis (Evans et al., 2019; Schneider et al., 2022). Jamestown Canyon virus was first isolated from a *Culiseta inornata* mosquito in Jamestown Canyon, Colorado in 1961 (Schneider et al., 2022). Since then, JCV has been isolated from over 26 species of mosquitoes

belonging to six different genera including *Aedes*, *Anopheles*, *Culex*, *Culiseta*, *Psorophora*, and *Coquillettidia* (Schneider et al., 2022). The suspected mammal hosts for JCV include white-tailed deer (*Odocoileus virginianus*), moose (*Alces alces*), elk (*Cervus elaphus*), and bison (*Bison bison*) (Schneider et al., 2022). On average, there are about 12 human cases per year of JCV, however, since 2011 there has been a significant surge in reported cases, in midwestern states like Wisconsin and Minnesota (Shepard & Armstrong, 2023). Unlike LACV, JCV affects all age groups, with a median age of 48 years, and ranging from 10-69 years (Shepard & Armstrong, 2023). Interestingly, multiple studies showed that cases that progress to neurological involvement were disproportionally male, 68% male overall for all reported cases, and 56% male in Wisconsin cases alone (Shepard & Armstrong, 2023). It is suspected that the rise in detectable cases of JCV are due to the virus being added to serological testing protocols starting in 2013 (Pastula et al., 2015; Pastula, Smith, Beckham, & Tyler, 2016; Shepard & Armstrong, 2023). There are no approved vaccines or antivirals for JCV (Pastula et al., 2016).

Cache Valley virus

Cache Valley virus is an *Orthobunyavirus* first isolated from a *Culiseta inornata* in Utah, in 1956 (Holden & Hess, 1959). Since its initial discovery, it has been associated with significant health effects in ruminants and rare cases in humans (Holden & Hess, 1959). In ruminants, CVV is known to cause abortion storms and significant birth defects, including arthrogryposis and musculoskeletal defects (Chung, Livingston, Edwards, Crandell, et al., 1990; Edwards, 1994). In humans, it is associated with flu like symptoms, including fever, headaches, nausea, fatigue, encephalitis, meningitis, spontaneous abortions, and macrocephaly in infants (Andreadis, Armstrong, Anderson, & Main, 2014; Calisher & Sever, 1995; Campbell et al., 2006; Nguyen et

al., 2013; Sexton et al., 1997; Wilson et al., 2017). Cache Valley virus has a wide geographic range spanning most of North America (Muller, López, Escobar, & Auguste, 2024). Cache Valley virus has a wide range of potential vectors, with 44 confirmed mosquito species from various genera including *Aedes*, *Anopheles*, *Coquillettidia*, *Culex*, *Culiseta*, *Mansonia*, and *Psorophora* (Andreadis et al., 2014; Waddell et al., 2019) . Cache Valley virus is likely to have a wide range of vertebrate host species such as white-tailed deer, mule deer (*Odocoileus hemionus*), elk (*Cervus elaphus*), swift foxes (*Vulpes velox*), kit foxes (*V. macrotus*), raccoons (*Procyon lotor*), eastern cottontails (*Sylvilagus floridanus*), and jackrabbits (*Lepus californicus*) (Aguirre, McLean, Cook, & Quan, 1992; Blackmore & Grimstad, 1998, 2008; Buescher et al., 1970; Eldridge, Calisher, Fryer, Bright, & Hobbs, 1987; D. Miller et al., 2000). Although, CVV has wide range of potential hosts and vectors, it is most often associated with ruminants (especially domestic sheep).

Given that most recorded CVV outbreaks occurred in domestic ovine species, the few animal models that have been described have used sheep (Edwards, Livingston, Chung, & Collisson, 1989), and of those, most involved pregnant subjects. Ovine fetuses have a narrow window of susceptibility to CVV, between 29 and 47 days of gestation. Before the susceptibility period fetuses are mummified and after this period animals have an immune system capable of circumventing infection (Chung, Livingston, Edwards, Crandell, et al., 1990; Chung, Livingston, Edwards, Gauer, & Collisson, 1990). The fetuses of experimentally infected sheep showed necrosis in the central nervous system (CNS) and skeletal muscle by 14 days post infection (DPI), and hydrocephalus, micromyelia, and muscular loss by 28 DPI (Rodrigues Hoffmann et al., 2013; Rodrigues Hoffmann et al., 2012). Other studies also showed that fetuses that developed malformations had CVV in the cytoplasm of both the brain and CNS but not in microglial cells even though there was marked microgliosis (Rodrigues Hoffmann et al., 2012). There are very few

studies investigating CVV pathogenesis in adult sheep. For instance, experimentally infected 10-month-old ewes did show signs of disease where 50% developed a fever, and 75% had detectable re-isolatable virus on 1 and 2 DPI (Chung, Livingston, Jones, & Collisson, 1991).

In addition to agricultural ruminant species, there have been a few attempts to determine wildlife species that may be used as animal models. Blackmore and Grimstad (2008) infected eastern cottontails (*S. floridanus*) and found that a very small proportion (4%) showed viremia. White-tailed deer were also infected with CVV and found that 100% became viremic for several days (Blackmore & Grimstad, 1998). A few other studies, infected a wide variety of domestic and wildlife species including: pigs, goats, sheep, dogs, cows, northern racoons, horses, groundhogs, Virginia opossums, mice, and hamsters, but were not able to induce disease or obtain detectable viremia (Kokernot et al., 1969).

To determine the distribution of CVV in North America, various serological surveys were conducted. As early as 1966, studies showed 12% seroprevalence for CVV in wild birds, and 14.3% in domestic chickens (Belle, Grant, & Griffiths, 1966). Additional surveys have showed that sheep demonstrate very high seropositivity for CVV, with 96.7% in the eastern US, 53.3% in central US, and 58.9% in the western US (Meyers et al., 2015). In humans, some studies have suggested seropositivity as high as 40% (Work, 1964). The true incidence of CVV, however, is still undetermined, and many researchers suggest that there are severe underreporting and misdiagnosis of the virus (Dimitrova et al., 2011; Muller et al., 2024).

Animal Models

Animal models have revolutionized the way biomedical science can be conducted and have led to insurmountable progress in many fields of study, but especially in virology (Bouvier &

Lowen, 2010). Animal models are an integral part of better understanding the pathogenesis of a disease and necessary for the development of interventions (Bouvier & Lowen, 2010). Although several animal models for CVV were previously established, these models only involved large animals such as sheep (Chung, Livingston, Edwards, Crandell, et al., 1990; Chung, Livingston, Edwards, Gauer, et al., 1990; Rodrigues Hoffmann et al., 2013; Rodrigues Hoffmann et al., 2012). Large animal models are extremely important, but have some significant limitations, including small sample sizes and issues with housing facilities, cost, and time. Previous attempts have failed to use mice and birds as models for CVV in laboratory research (Holden & Hess, 1959; Waddell et al., 2019). Herein, I developed a mouse model, using IFNAR$^{-/-}$ mice, that efficiently replicates the natural progression of disease observed during CVV infection. This animal model will be able to shed light on the progression of CVV-induced disease and will also serve as an integral tool for the development and testing of vaccines and therapeutics for CVV.

The State of Virginia has a very large agricultural sector, which overall contributes approximately 13.6 billion dollars annually to the Commonwealth (Virginia Poultry Federation, 2024). Virginia houses more than 800 chicken farms state-wide, and over 275 turkey farms (Virginia Poultry Federation, 2024). As a large part of the gross domestic product in the state, knowing and maintaining the health of these animals is critical to maintaining the profitability of the industry. For example, during influenza outbreaks, entire chicken colonies can be euthanized to reduce the spread of the disease (Seeger, Hagerman, Johnson, Pendell, & Marsh, 2021). These influenza outbreaks cost millions of dollars to farmers nationally (Seeger et al., 2021). Although, we know a lot about the economic cost and effects of influenza virus on poultry in the US and Virginia, we know very little about the effects of arboviruses on poultry. The C.D.C. guidelines for arbovirus testing in poultry, is limited to only a handful of viruses which include: EEEV,

Western Equine Encephalitis, and St. Louis Encephalitis (Moore et al., 1993). Since the early 2000s, very little screening has been conducted focused on birds, outside of WNV and EEEV, in Virginia (Loftin, Herbert, & Phaltankar, 2006; E. McDonald, 2021). Previous studies on migratory birds and sentinel poultry have demonstrated that birds are exposed to arboviruses and can carry a wide variety of viruses, including CVV (Belle et al., 1966; McLean & Ubico, 2007; Reed, Meece, Henkel, & Shukla, 2003). To better understand the role that birds may play in CVV transmission and distribution, I assessed various avian species for CVV infection and disease. A chicken model was previously attempted in the late 1950s with little success (Holden & Hess, 1959). My poultry studies showed that avian cell lines can efficiently grow CVV, but when poultry species were infected in a laboratory setting, they failed to induce an infection.

Arbovirus Surveillance

The recognition and importance of arboviral surveillance is relatively recent when compared to the surveillance of non-arboviral diseases. It was not until the early 1900s when Walter Reed, based on previous work conducted by Carlos Finlay, was able to determine that the transmission of yellow fever in Cuba was carried out by a mosquito vector (Gianchecchi, Cianchi, Torelli, & Montomoli, 2022). Their work inspired many others and led to the revolutionary "golden age" of arbovirology in the 1950s (Morens, 2023). During this time, the development of screening techniques, virus isolation, serology, and vaccine development completely changed the game when it came to viral discovery and disease tracking (Morens, 2023). This time in history cemented the importance of surveillance and developed an arsenal of tools that we continue to use today to study, track, and prevent the spread of arboviruses.

Mosquito surveillance is a critical component of public health, disease management, and vector control (Dacko, Nava, Vitek, & Debboun, 2020; Schwab, Stone, Fonseca, & Fefferman, 2018). As mentioned previously, arboviruses are responsible for millions of cases annually and can have long-lasting sequalae in patients with neuroinvasive disease (Pastula et al., 2016). Having baseline knowledge of the mosquitoes, their existing habitat range, and the viruses present in a particular area, allows for the development of better risk assessment and emergency outbreak strategies. Monitoring of mosquito populations also provides data of vector abundance and distribution, which is a critical aid for vector population control efforts targeting species that are likely viral vectors (Dacko et al., 2020; Schwab et al., 2018). Surveillance is a vital part of establishing effective and long-term vector control and allows for adjustments and optimizations based on changes in mosquito populations or viral distribution over time. Climate change has already had a significant effect on mosquito distribution (Armstrong, Andreadis, Shepard, & Thomas, 2017; Dacko et al., 2020; Rochlin et al., 2013), surveillance is invaluable tool that enables us to determine vector distributions and breeding patterns for vectors with expanding geographic ranges. Using surveillance as a public health tool allows for early detection of changes in patterns, which facilitates rapid response times and enables necessary adjustments based on public health need and novel pathogen threats. Finally, surveillance data can be used to inform and educate the public about the risks of mosquito borne diseases. It can increase community participation in control and prevention efforts (i.e., removing standing water sources and using personal protection measures).

The Commonwealth of Virginia is composed of five ecoregions, the coastal plains, piedmont, Blue ridge, Valley and Ridge, and the Appalachian plateau, making its geography incredibly diverse (Virginia Museum of History & Culture, 2024). The elevation range of Virginia

goes from sea level in the Tidewater rivers east to 1746 meters above sea level at Mount Rogers in Grayson country (Virginia Museum of History & Culture, 2024). This elevational gradient provides us a great opportunity to observe *in situ* how changes in geography affect the dispersal of disease vectors, in particular mosquitoes. The Virginia Department of Health conducts annual mosquito surveys, which are limited both in location (cities: Virginia Beach, Richmond, and Fairfax) and the viruses screened (i.e., WNV or EEEV) (Virginia Department of Health, 2019). Our knowledge of state-wide arbovirus prevalence and distribution of mosquitoes is lacking. With changes in climate and expanding vector ranges it is imperative that we conduct comprehensive surveys that can better inform healthcare officials and aid in the development of risk assessments.

For this reason, my dissertation has two main aims. The first is the development of animal models for the study of CVV pathogenesis in mice and poultry. The second addresses surveillance of mosquitoes and arboviruses across Virginia to assess the presence, emergence and re-emergence of Orthobunyaviruses in Virginia.

References

Aguirre, A. A., McLean, R. G., Cook, R. S., & Quan, T. J. (1992). Serologic survey for selected arboviruses and other potential pathogens in wildlife from Mexico. *J Wildl Dis, 28*(3), 435-442.

Amanna, I. J., & Slifka, M. K. (2020). Successful vaccines. *Vaccination strategies against highly variable pathogens*, 1-30.

Andreadis, T., Armstrong, P., Anderson, J., & Main, A. (2014). Spatial-temporal analysis of Cache Valley virus (*Bunyaviridae: Orthobunyavirus*) infection in anopheline and culicine mosquitoes (Diptera: Culicidae) in the northeastern United States, 1997-2012. *Vector Borne Zoonotic Dis, 14*(10), 763-773.

Armstrong, P. M., Andreadis, T. G., Shepard, J. J., & Thomas, M. C. (2017). Northern range expansion of the Asian tiger mosquito (*Aedes albopictus*): analysis of mosquito data from Connecticut, USA. *PLoS Neglected tropical diseases, 11*(5), e0005623.

Barr, J., Weber, F., & Schmaljohn, C. (2020). *Bunyavirales:* The viruses and their replication, *Fields virology: Emerging viruses* (Vol. 7th Edition pp. 706-749). Lippincott Williams & Wilkins, Philadelphia, PA, USA.

Belle, E. A., Grant, L. S., & Griffiths, B. B. (1966). The isolation of Cache Valley virus from mosquitoes in Jamaica. *West Indian Med J, 15*(4), 217-220.

Belshaw, R., Pybus, O. G., & Rambaut, A. (2007). The evolution of genome compression and genomic novelty in RNA viruses. *Genome research, 17*(10), 1496-1504.

Blackmore, C., & Grimstad, P. (1998). Cache Valley and Potosi viruses (*Bunyaviridae*) in white-tailed deer (*Odocoileus virginianus*): experimental infections and antibody prevalence in natural populations. *Am J Trop Med Hyg, 59*(5), 704-709.

Blackmore, C., & Grimstad, P. (2008). Evaluation of the eastern cottontail *Sylvilagus floridanus* as an amplifying vertebrate host for Cache Valley virus (*Bunyaviridae*) in Indiana. *J Wildl Dis, 44*(1), 188-192.

Bouvier, N. M., & Lowen, A. C. (2010). Animal models for influenza virus pathogenesis and transmission. *Viruses, 2*(8), 1530-1565.

Buescher, E. L., Byrne, R. J., Clarke, G. C., Gould, D. J., Russell, P. K., Scheider, F. G., & Yuill, T. M. (1970). Cache Valley virus in the Del Mar Va Peninsula. I. Virologic and serologic evidence of infection. *Am J Trop Med Hyg, 19*(3), 493-502.

Bursali, F., & Simsek, F. M. (2024). Effects of different feeding methods and hosts on the fecundity and blood-feeding behavior of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae). *Biologia, 79*(8), 2423-2431.

Bushman, F. D., McCormick, K., & Sherrill-Mix, S. (2019). Virus structures constrain transmission modes. *Nature microbiology, 4*(11), 1778-1780.

C.D.C., Centers for Disease Control (2024). La Crosse Virus, Retrieved from https://www.cdc.gov/la-crosse-encephalitis/data-maps/index.html. Accessed on 26 December 26, 2024.

Calisher, C. H., & Sever, J. L. (1995). Are North American Bunyamwera serogroup viruses etiologic agents of human congenital defects of the central nervous system? *Emerg Infect Dis, 1*(4), 147-151.

Campbell, G. L., Mataczynski, J. D., Reisdorf, E. S., Powell, J. W., Martin, D. A., Lambert, A. J., . . . Lanciotti, R. S. (2006). Second Human Case of Cache Valley Virus Disease. *Emerging Infectious Diseases, 12*(5).

Chung, S., Livingston, C., Edwards, J., Crandell, R., Shope, R., Shelton, M., & Collisson, E. (1990). Evidence that Cache Valley Virus Induces Congenital Malformations in Sheep. *Veterinary Microbiology, 21*.

Chung, S., Livingston, C., Jr., Jones, C., & Collisson, E. (1991). Cache Valley virus infection in Texas sheep flocks. *Journal of the American Veterinary Medical Association, 199*(3), 337-340.

Chung, S., Livingston, C., Jr., Edwards, J., Gauer, B., & Collisson, E. (1990). Congenital malformations in sheep resulting from in utero inoculation of Cache Valley virus. *Am J Vet Res, 51*(10), 1645-1648.

da Silva Pessoa Vieira, C. J., Steiner São Bernardo, C., Ferreira da Silva, D. J., Rigotti Kubiszeski, J., Serpa Barreto, E., de Oliveira Monteiro, H. A., . . . Levi, T. (2022). Land-use effects on mosquito biodiversity and potential arbovirus emergence in the Southern Amazon, Brazil. *Transboundary and Emerging Diseases, 69*(4), 1770-1781.

Dacko, N. M., Nava, M. R., Vitek, C., & Debboun, M. (2020). Mosquito surveillance. *Mosquitoes, Communities, and Public Health in Texas* (pp. 221-247)

Dimitrova, K., Andonova, M., Makowski, K., Holloway, K., Levett, P., Kadkhoda, K., & Drebot, M. (2011). Preliminary evidence of Cache Valley virus infections and associated human illness in western Canada in 2009. *Can J Infect Dis Med Microbiol, 22*, 15A.

Durden, L. A., & Mullen, G. R. (2019). Introduction: Medical-Veterinary Entomology, *Medical and Veterinary Entomology* (3rd Edition pp. 1-10). Academic Press, Cambridge, MA, USA

Edwards, J. F. (1994). Cache Valley Virus. *Veterinary Clinics of North America: Food Animal Practice, 10*(3), 515-524.

Edwards, J. F., Livingston, C. W., Chung, S. I., & Collisson, E. C. (1989). Ovine arthrogryposis and central nervous system malformations associated with in utero Cache Valley virus infection: spontaneous disease. *Vet Pathol, 26*(1), 33-39.

Eldridge, B. F., Calisher, C. H., Fryer, J. L., Bright, L., & Hobbs, D. J. (1987). Serological evidence of California serogroup virus activity in Oregon. *J Wildl Dis, 23*(2), 199-204.

Elliott, R. M. (2014). *Orthobunyaviruses*: recent genetic and structural insights. *Nature Reviews Microbiology, 12*(10), 673-685.

Escobar, L. E., Qiao, H., & Peterson, A. T. (2016). Forecasting Chikungunya spread in the Americas via data-driven empirical approaches. *Parasites & Vectors, 9*, 1-12.

Evans, A. B., Winkler, C. W., & Peterson, K. E. (2019). Differences in Neuropathogenesis of Encephalitic California Serogroup Viruses. *Emerg Infect Dis, 25*(4), 728-738.

Fleischmann, W. (1996). Viral Genetics, *Medical Microbiology. 4th edition*, University of Texas Medical Branch at Galveston, Galveston, TX, USA.

Foster, W. A., & Walker, E. D. (2019). Mosquitoes (*Culicidae*). *Medical and Veterinary Entomology* (Vol. 3rd Edition pp. 261-317). Academic Press, Cambridge, MA, USA

Gianchecchi, E., Cianchi, V., Torelli, A., & Montomoli, E. (2022). Yellow Fever: Origin, Epidemiology, Preventive Strategies and Future Prospects. *Vaccines, 10*(3), 372.

Gillim-Ross, L., & Subbarao, K. (2006). Emerging respiratory viruses: challenges and vaccine strategies. *Clinical microbiology reviews, 19*(4), 614-636.

Griffin, D., & Weaver, S. (2020). *Alphaviruses*, *Fields virology: Emerging viruses* (Vol. 7, pp. 194-245). Lippincott Williams & Wilkins., Philadelphia, PA, USA.

Harding, S., Greig, J., Mascarenhas, M., Young, I., & Waddell, L. (2019). La Crosse virus: A scoping review of the global evidence. *Epidemiology & Infection, 147*, e66.

Hartman, A. L., & Myler, P. J. (2023). *Bunyavirales*: scientific gaps and prototype pathogens for a large and diverse group of zoonotic viruses. *The Journal of Infectious Diseases, 228*(Supplement_6), S376-S389.

Holden, P., & Hess, A. D. (1959). Cache Valley virus, a previously undescribed mosquito-borne agent. *Science, 130*(3383), 1187-1188.

Kokernot, R. H., Hayes, J., Tempelis, C. H., Chan, D. H., Boyd, K. R., & Anderson, R. J. (1969). Arbovirus studies in the Ohio-Mississippi Basin, 1964-1967. IV. Cache Valley virus. *Am J Trop Med Hyg, 18*(5), 768-773.

Koonin, E., & Yutin, N. (2019). Evolution of the Large Nucleocytoplasmic DNA Viruses of Eukaryotes and Convergent Origins of Viral Gigantism. *Advances in Virus Research* (Vol. 103, pp. 167-202): Academic Press, Cambridge, MA, USA

Lechevalier, H. (1972). Dmitri Iosifovich Ivanovski (1864-1920). *Bacteriological reviews, 36*(2), 135-145.

Lesbarrères, D., Balseiro, A., Brunner, J., Chinchar, V., Duffus, A., Kerby, J., . . . Waltzek, T. (2012). Ranavirus: past, present and future. In: *The Royal Society*, 8(4), 481-483.

Loftin, K. C., Herbert, M. W., & Phaltankar, P. G. (2006). Five-year surveillance of West Nile and eastern equine encephalitis viruses in southeastern Virginia. *Journal of environmental health, 68*(9), 33.

Louten, J. (2016). Virus Structure and Classification. *Essential human virology*, 19.

Madewell, Z. J. (2020). Arboviruses and their vectors. *Southern Medical Journal, 113*(10), 520.

McDonald, E. (2021). Surveillance for West Nile virus disease—United States, 2009–2018. *MMWR. Surveillance Summaries, 70*.

McDonald, S., Nelson, M., Turner, P., & Patton, J. (2016). Reassortment in segmented RNA viruses: mechanisms and outcomes. *Nature Reviews Microbiology, 14*(7), 448-460.

McLean, R. G., & Ubico, S. R. (2007). Arboviruses in Birds. *Infectious diseases of wild birds* (pp. 17-62).

Meyers, M. T., Bahnson, C. S., Hanlon, M., Kopral, C., Srisinlapaudom, S., Cochrane, Z. N., . . . Blitvich, B. J. (2015). Management Factors Associated with Operation-Level Prevalence of Antibodies to Cache Valley Virus and Other Bunyamwera Serogroup Viruses in Sheep in the United States. *Vector Borne Zoonotic Dis, 15*(11), 683-693.

Miller, B. (2008). Arboviruses, *Encyclopedia of Virology (Third Edition)* (pp. 170-176). Academic Press, Cambridge, MA, USA

Miller, B., DeFoliart, G., & Yuill, T. (1977). Vertical transmission of La Crosse virus (California encephalitis group): transovarial and filial infection rates in *Aedes triseriatus* (Diptera: Culicidae). *Journal of Medical Entomology, 14*(4), 437-440.

Miller, D., Covell, D., McLean, R., Adrian, W., Niezgoda, M., Gustafson, J., . . . Quan, T. (2000). Serologic survey for selected infectious disease agents in swift and kit foxes from the western United States. *J Wildl Dis, 36*(4), 798-805.

Moore, C. G., Marfin, A. A., Mitchell, C. J., McLean, R. G., Calisher, C. H., Tsai, T. F., . . . Moore, P. (1993). Guidelines for arbovirus surveillance programs in the United States.

Morens, D. M. (2023). The "Golden Age" of Arbovirology, 1950–1969, *History of Arbovirology: Memories from the Field: Volume I: Personal Reflections* (pp. 381-423). Cham: Springer International Publishing, New York, NY, USA

Mueller, S. N., & Rouse, B. T. (2009). Immune responses to viruses. *Clinical Immunology*, 421.

Muller, J. A., López, K., Escobar, L. E., & Auguste, A. J. (2024). Ecology and geography of Cache Valley virus assessed using ecological niche modeling. *Parasites & Vectors, 17*(1), 270.

Nathanson, N. (2016). The Human Toll of Viral Diseases: Past Plagues and Pending Pandemics. *Viral Pathogenesis (Third Edition)* (pp. 3-16). Academic Press, Cambridge, MA, USA

Nguyen, N. L., Zhao, G., Hull, R., Shelly, M. A., Wong, S. J., Wu, G., . . . Menegus, M. A. (2013). Cache valley virus in a patient diagnosed with aseptic meningitis. *J Clin Microbiol, 51*(6), 1966-1969.

Pastula, D., Johnson, D. H., White, J., Dupuis, A., Fischer, M., & Staples, J. (2015). Jamestown Canyon virus disease in the United States—2000–2013. *The American journal of tropical medicine and hygiene, 93*(2), 384.

Pastula, D., Smith, D., Beckham, J., & Tyler, K. (2016). Four emerging arboviral diseases in North America: Jamestown Canyon, Powassan, chikungunya, and Zika virus diseases. *J Neurovirol, 22*(3), 257-260.

Payne, S. (2017a). Introduction to Animal Viruses. *Viruses* (pp. 1-10). Academic Press, Cambridge, MA, USA

Payne, S. (2017b). Introduction to DNA Viruses. *Viruses* (pp. 231-236): Academic Press, Cambridge, MA, USA

Payne, S. (2017c). Introduction to RNA Viruses. *Viruses* (pp. 97-105): Academic Press, Cambridge, MA, USA

Pierson, T., Lazear, H., & Diamond, M. (2020). *Flaviviruses:* Dengue, Zika, West Nile, Yellow Fever and other Flaviviruses. *Fields virology: Emerging viruses* (Vol. 7, pp. 345-409). Lippincott Williams & Wilkins, Philadelphia, PA, USA.

Qureshi, A. (2018). Chapter 2 - Mosquito-Borne Diseases. *Zika Virus Disease* (pp. 27-45). Academic Press, Cambridge, MA, USA

Reed, K. D., Meece, J. K., Henkel, J. S., & Shukla, S. K. (2003). Birds, migration and emerging zoonoses: west nile virus, lyme disease, influenza A and enteropathogens. *Clin Med Res, 1*(1), 5-12.

Robert, M. A., Stewart-Ibarra, A. M., & Estallo, E. L. (2020). Climate change and viral emergence: evidence from *Aedes*-borne arboviruses. *Current opinion in virology, 40*, 41-47.

Rochlin, I., Ninivaggi, D., Hutchinson, M., & Farajollahi, A. (2013). Climate change and range expansion of the Asian tiger mosquito (*Aedes albopictus*) in Northeastern USA: implications for public health practitioners. *PLoS One, 8*(4), e60874.

Rodrigues Hoffmann, A., Dorniak, P., Filant, J., Dunlap, K. A., Bazer, F. W., de la Concha-Bermejillo, A., . . . Edwards, J. F. (2013). Ovine fetal immune response to Cache Valley virus infection. *Journal of virology, 87*(10), 5586-5592.

Rodrigues Hoffmann, A., Welsh, C. J., Varner, P. W., de la Concha-Bermejillo, A., Ball, J. M., Ambrus, A., & Edwards, J. F. (2012). Identification of the target cells and sequence of infection during experimental infection of ovine fetuses with Cache Valley virus. *Journal of virology, 86*(9), 4793-4800.

Roiz, D., Pontifes, P. A., Jourdain, F., Diagne, C., Leroy, B., Vaissiere, A. C., . . . Courchamp, F. (2024). The rising global economic costs of invasive *Aedes* mosquitoes and *Aedes*-borne diseases. *Sci Total Environ, 933*, 173054.

Schneider, E. F., Robich, R. M., Elias, S. P., Lubelczyk, C. B., Cosenza, D. S., & Smith, R. P. (2022). Jamestown Canyon Virus in Collected Mosquitoes, Maine, United States, 2017-2019. *Emerg Infect Dis, 28*(11), 2330-2333.

Schwab, S. R., Stone, C. M., Fonseca, D. M., & Fefferman, N. H. (2018). The importance of being urgent: the impact of surveillance target and scale on mosquito-borne disease control. *Epidemics, 23*, 55-63.

Seeger, R., Hagerman, A., Johnson, K., Pendell, D., & Marsh, T. (2021). When poultry take a sick leave: Response costs for the 2014–2015 highly pathogenic avian influenza epidemic in the USA. *Food Policy, 102*, 102068.

Sexton, D. J., Rollin, P. E., Breitschwerdt, E. B., Corey, G. R., Myers, S. A., Dumais, M. R., . . . Ksiazek, T. G. (1997). Life-Threatening Cache Valley Virus Infection. *The New England Journal of Medicine, 336*(8).

Shepard, J. J., & Armstrong, P. M. (2023). Jamestown Canyon virus comes into view: understanding the threat from an underrecognized arbovirus. *Journal of Medical Entomology, 60*(6), 1242-1251.

Spiropoulou, C., & Bente, D. (2020). *Orthohantavirus*, *Orthonairovirus*, *Orthobunyavirus* and *Phlebovirus*, *Fields virology: Emerging viruses* (Vol. 7th Edition pp. 750-783). Lippincott Williams & Wilkins, London, UK.

Sullivan, M. F., Gould, D. J., & Maneechai, S. (1971). Observations on the host range and feeding preferences of *Aedes albopictus* (Skuse). *Journal of Medical Entomology, 8*(6), 713-716.

Szpara, M. L., & Van Doorslaer, K. (2021). Mechanisms of dna virus evolution. *Encyclopedia of Virology*, 71.

Tsetsarkin, K., McGee, C. E., Volk, S. M., Vanlandingham, D. L., Weaver, S. C., & Higgs, S. (2009). Epistatic roles of E2 glycoprotein mutations in adaption of chikungunya virus to *Aedes albopictus* and *Ae. aegypti* mosquitoes. *PLoS One, 4*(8), e6835.

Tsetsarkin, K., Vanlandingham, D., McGee, C., & Higgs, S. (2007). A single mutation in chikungunya virus affects vector specificity and epidemic potential. *PLoS Pathogens, 3*(12), e201.

Virginia Department of Health. (2019). *VDH Monthly Mosquito Activity Report* Retrieved from https://www.vdh.virginia.gov/?s=VDH+monthly+mosquito+activity+report#gsc.tab=0&gsc.q=VDH%20monthly%20mosquito%20activity%20report&gsc.page=1, Accessed December 26, 2024

Virginia Museum of History & Culture. (2024). The Regions of Virginia Retrieved from https://virginiahistory.org/learn/regions-virginia, Accessed December 26, 2024

Virginia Poultry Federation. (2024). Virginia Poultry Industry Facts and Figures. Retrieved from https://www.vapoultry.com/resources/facts.cfm, Accessed December 26, 2024

World Health Organization, W.H.O., The Top 10 Causes of Death Retrieved from https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death#:~:text=Leading%20causes%20of%20death%20in,9.1%20million%20deaths%20in%202021, Accessed August 7th, 2024

Waddell, L., Pachal, N., Mascarenhas, M., Greig, J., Harding, S., Young, I., & Wilhelm, B. (2019). Cache Valley virus: A scoping review of the global evidence. *Zoonoses Public Health, 66*(7), 739-758.

Wilson, M., Suan, D., Duggins, A., Schubert, R., Khan, L., Sample, H., . . . DeRisi, J. (2017). A novel cause of chronic viral meningoencephalitis: Cache Valley virus. *Ann Neurol, 82*(1), 105-114.

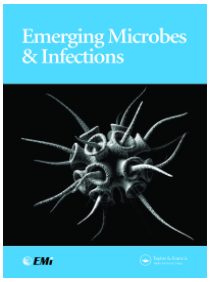
Work, T. H. (1964). Serological evidence of arbovirus infection in the Seminole Indians of southern Florida. *Science, 145*(3629), 270-272.

Chapter 1: Novel murine models for studying Cache Valley virus pathogenesis and *in utero* transmission

Authors: Krisangel López[1], Sarah N. Wilson[1], Sheryl Coutermash-Ott[2], Manette Tanelus[1], William B. Stone[1], Danielle L. Porier[1], Dawn I. Auguste[1], John A. Muller[3], Lin Kang[4], Sally L. Paulson[1], Jesse H. Erasmus[5], Albert J. Auguste[1,6*]

Author Affiliations: [1]Department of Entomology, College of Agriculture and Life Sciences, Fralin Life Science Institute, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061; [2]Department of Biomedical Sciences and Pathobiology, Virginia Tech, VA-MD College of Veterinary Medicine, Blacksburg, VA, 24061; [3]Department of Biology, University of Oklahoma, 660 Parrington Oval, Norman, OK, 73019; [4]Edward Via College of Osteopathic Medicine, Monroe, LA, 71203; [5]HDT Bio, Seattle, WA 98102; [6]Center for Emerging, Zoonotic, and Arthropod-borne Pathogens, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061.

I (Krisangel López) am first author of the following manuscript, which is published by Informa UK Limited, trading as Taylor & Francis Group, on behalf of Shanghai Shangyixun Cultural Communication Co., Ltd. **Published:** ***Emerging Microbes and Infections***
DOI:10.1080/22221751.2021.1965497



Taylor & Francis
Taylor & Francis Group

Emerging Microbes & Infections

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/temi20

Novel murine models for studying Cache Valley virus pathogenesis and *in utero* transmission

Krisangel López, Sarah N. Wilson, Sheryl Coutermash-Ott, Manette Tanelus, William B. Stone, Danielle L. Porier, Dawn I. Auguste, John A. Muller, Orchid M. Allicock, Sally L. Paulson, Jesse H. Erasmus & Albert J. Auguste

To cite this article: Krisangel López, Sarah N. Wilson, Sheryl Coutermash-Ott, Manette Tanelus, William B. Stone, Danielle L. Porier, Dawn I. Auguste, John A. Muller, Orchid M. Allicock, Sally L. Paulson, Jesse H. Erasmus & Albert J. Auguste (2021) Novel murine models for studying Cache Valley virus pathogenesis and *in utero* transmission, Emerging Microbes & Infections, 10:1, 1649-1659, DOI: 10.1080/22221751.2021.1965497

To link to this article: https://doi.org/10.1080/22221751.2021.1965497

Emerging Microbes & Infections
2021, VOL. 10
https://doi.org/10.1080/22221751.2021.1965497

Novel murine models for studying Cache Valley virus pathogenesis and *in utero* transmission

Krisangel López[a], Sarah N. Wilson[a], Sheryl Coutermash-Ott[b], Manette Tanelus[a], William B. Stone[a], Danielle L. Porier[a], Dawn I. Auguste[a], John A. Muller[c], Orchid M. Allicock[d], Sally L. Paulson[a], Jesse H. Erasmus[e] and Albert J. Auguste [a,f]

[a]Department of Entomology, College of Agriculture and Life Sciences, Fralin Life Science Institute, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA; [b]Department of Biomedical Sciences and Pathobiology, Virginia Tech, VA-MD College of Veterinary Medicine, Blacksburg, VA, USA; [c]Department of Biology, University of Oklahoma, Norman, OK, USA; [d]Department of Epidemiology of Microbial Diseases, Yale School of Public Health, New Haven, CT, USA; [e]HDT Bio, Seattle, WA, USA; [f]Center for Emerging, Zoonotic, and Arthropod-borne Pathogens, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA

ABSTRACT

Cache Valley virus (CVV) is a prevalent emerging pathogen of significant importance to agricultural and human health in North America. Emergence in livestock can result in substantial agroeconomic losses resulting from the severe embryonic lethality associated with infection during pregnancy. Although CVV pathogenesis has been well described in ruminants, small animal models are still unavailable, which limits our ability to study its pathogenesis and perform preclinical testing of therapeutics. Herein, we explored CVV pathogenesis, tissue tropism, and disease outcomes in a variety of murine models, including immune -competent and -compromised animals. Our results show that development of CVV disease in mice is dependent on innate immune responses, and type I interferon signalling is essential for preventing infection in mice. IFN-αβR$^{-/-}$ mice infected with CVV present with significant disease and lethal infections, with minimal differences in age-dependent pathogenesis, suggesting this model is appropriate for pathogenesis-related, and short- and long-term therapeutic studies. We also developed a novel CVV *in utero* transmission model that showed high rates of transmission, spontaneous abortions, and congenital malformations during infection. CVV infection presents a wide tissue tropism, with significant amplification in liver, spleen, and placenta tissues. Immune-competent mice are generally resistant to infection, and only show disease in an age dependent manner. Given the high seropositivity rates in regions of North America, 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.

ARTICLE HISTORY Received 20 April 2021; Revised 16 July 2021; Accepted 4 August 2021

KEYWORDS Cache Valley virus; pathogenesis; animal models; murine model; orthobunyavirus

Introduction

Cache Valley virus (CVV) is an emerging zoonotic mosquito-borne virus, and a member of the Bunyamwera serogroup along with other veterinary and public health important viruses within the genus *Orthobunyavirus* [1–3]. Since CVV's first isolation in 1956, in Cache Valley, Utah [4] it has become an important pathogen in Canada, the U.S., and Mexico, where its emergence has resulted in significant agroeconomic losses. Although CVV emergence has historically been sporadic, it remains an important public health and agricultural concern as exemplified by its recent outbreaks in Canada in 2016 and New York in 2021 [5,6].

CVV virions are enveloped, pleomorphic, and ~80–120 nm in diameter. Virions encapsidate a tri-segmented, negative sense genome that includes the L segment which encodes a viral RNA polymerase, an M segment that encodes the glycoproteins Gn and Gc and a nonstructural protein (NSm), and an S segment that encodes the nucleocapsid and a small nonstructural protein (NSs) [7]. Currently, there are two phylogenetic lineages of CVV, an ancestral lineage I (1952–2011), and the most recent lineage II (2011–2014) which is thought to have emerged from a Mexican strain isolated in the Yucatan peninsula [8]. Since the discovery of the new lineage II strain, subsequent surveillance studies demonstrate nearly all samples collected in Connecticut in 2014 (i.e. four years after it was identified) are lineage II strains, suggesting a potential replacement event [8].

CVV has an extraordinarily wide host range and has previously been serologically detected or isolated from several domestic animals including sheep (*Ovis*

CONTACT Albert J. Auguste jauguste@vt.edu Department of Entomology, Fralin Life Science Institute, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA

Supplemental data for this article can be accessed at https://doi.org/10.1080/22221751.2021.1965497

aries), goats (*Capra hircus*), cattle (*Bos taurus)*, horses (*Equus caballus*), swine (*Sus scrofus*), rabbits (family Leporidae), and guinea pigs (*Cavia porcellus*) [7,9]. There is strong evidence that CVV transmission can be maintained in horses (*Equus caballus*) [10], swine, goats, [11], elk (*Cervus canadensis*) [12], and white-tailed deer (*Odocoileus virginianus*) which are suspected to be the primary amplification host [13,14]. Serological surveys conducted on sheep throughout the U.S. have demonstrated high seropositivity rates for CVV. Rates were as high as 96.7% in the eastern U.S., to 53.3% in the central U.S., and 58.9% in the Western U.S. [15]. CVV infections have been reported in humans [9,16–19] and seroprevalence rates in humans have been shown to be as high as 40% [9,19–22]. The virus is known to cause disease in humans, including fever, headaches, nausea, fatigue, encephalitis, meningitis, spontaneous abortions, and macrocephaly in infants [17–20,23,24]. A unique and critical phenotype underlying the importance of CVV is its teratogenicity. CVV infection during pregnancy causes a range of congenital birth defects in small ruminants, including reproductive failure and/or major congenital defects of the skeletal and central nervous systems [9,11,24–26].

CVV demonstrates a rare vector transmissibility phenotype, and is maintained through horizontal transmission, with some evidence of vertical transmission in multiple mosquito genera, including *Aedes, Anopheles, Culiseta,* and *Coquillettidia* species [27–29]. This has enormous ramifications since these vectors opportunistically feed on most livestock, and/or humans in their proximity. The close association of these vectors with current agricultural practices combined with CVV's high prevalence in nature, suggest a high risk of infection and epizootic/epidemic potential in North America [5].

CVV is an important pathogen and intervention strategies are urgently needed to prevent its emergence. Despite its significance, there are very limited resources and currently no available murine models for studying CVV pathogenesis or evaluating countermeasures. Here, we explored CVV pathogenesis, tissue tropism and disease outcomes in a variety of murine models, including immune -competent and -compromised animals. We also compare the pathogenesis among contemporaneous isolates of both CVV lineages to identify which strain should be preferentially selected as a challenge model for countermeasure testing. Our studies show that development of CVV disease in mice is dependent on innate immune responses and type I interferon signalling is essential for preventing infection in mice. Among the models studied, we also develop a CVV *in utero* transmission model that shows high rates of transmission and spontaneous abortions during infection. Altogether, our novel murine models demonstrate that CVV pathogenesis is type I interferon-dependent, as well as age-dependent in immune-competent mice.

Materials and methods

Viruses and cell culture

CVV strains from both genetic lineages were obtained from Dr. Sally Paulson and Dr. Philip Armstrong. Viruses were selected to represent CVV's genetic diversity, ensure they were isolated from different enzootic sources, and have minimal passage histories to reduce cell culture adaptive effects (Table S1). Viruses were propagated in Vero-76 (African Green Monkey Kidney) and C6/36 (*Ae. albopictus*) cells (ATCC; Manassas, VA, USA) and titers are shown in Table S1. Briefly, 80% confluent cell monolayers were inoculated with an aliquot of each virus strain, and cultures were harvested when 50% cytopathic effects (CPE) were observed in Vero-76 cells. Culture supernatants were collected, clarified by centrifugation at 3345 × g for ten minutes at 4°C and stored at −80° C. Virus titers were estimated by plaque assays on Vero-76 cells as previously described [30]. Complete genome sequences for 4B and W08491 were determined using next-generation sequencing as previously described [31], and sequences are available in GenBank under accession numbers MZ612419, MZ612420 and MZ612423 for 4B and MZ612418, MZ612421 and MZ612422 for W08491.

CVV pathogenesis in immune-compromised IFN-αβR$^{-/-}$ mice

Six-week-old IFN-αβR$^{-/-}$ (interferon alpha and beta receptor 1 knockout on a BL6 background) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA; Mouse strain: B6 129S2 -*ifnar1*^{tm1Agt}/Mmjax) and a colony maintained at Virginia Tech. Mice were separated into three groups including CVV 4B ($n = 14$), CVV W08491 ($n = 14$), and phosphate buffered saline (PBS) ($n = 13$; unchallenged healthy controls) and inoculated subcutaneously with 10^4 plaque forming units (PFU) of CVV, or with PBS diluent. Mice were inoculated subcutaneously as previously done with ruminants and to reflect a natural peripheral infection route [32]. Mice were bled retro-orbitally for the first four days post infection (DPI) to assess viremia. Blood samples were separated by centrifugation at 3099 × g for five minutes, and serum samples were labelled and stored at −80°C until plaque assays were performed. After challenge, mice were monitored for weight change, signs of disease, and mortality for 14 days. Necropsies were conducted on 3 mice per group on 3 and 5 DPI. Tissues were collected, half of which were fixed in 10% formalin solution for histological analysis, and the

other half stored in culture media (i.e. Dulbecco's Modified Eagle Media (DMEM) containing 2% Fetal Bovine Serum (FBS), 100 units of Penicillin and 0.1 mg Streptomycin), for virus quantification by plaque assay. Mice were immediately euthanized when moribund or upon weight loss greater than or equal to 20% of their original body weight, as previously described [33].

Three-week old mice were separated into three groups CVV 4B ($n = 9$), CVV W08491 ($n = 9$), and PBS ($n = 9$; unchallenged healthy controls). Mice were inoculated, bled, monitored, and euthanized as above except that this study lasted 10 days. Necropsies and tissue collections were done on 3 DPI, and tissues processed as above.

One-year old mice were separated into three groups CVV 4B ($n = 12$), CVV W08491 ($n = 12$) and PBS ($n = 8$; unchallenged healthy controls). Mice were challenged with 10^4 PFU of CVV or PBS diluent. Mice were bled retro-orbitally on 2, 3, 4 and 5 DPI to assay for viremia. After challenge, weight change, mortality and signs of disease were monitored for 6 days. Necropsies were performed on 2, 4, and 6 DPI, with half of the tissues fixed in 10% formalin solution for histological analysis, and the other half stored in media for virus quantification as above.

In utero transmission of CVV in IFN-αβR$^{-/-}$ mice

Mice were separated into three groups CVV 4B ($n = 8$, CVV W08491 ($n = 9$) and PBS ($n = 7$). Adult IFN-αβR$^{-/-}$ dams were mated and monitored daily for vaginal plugs. Upon detection of vaginal plugs, embryonic development day E0.5 was noted. Mice were challenged subcutaneously ten days later (E10.5) with 10^4 PFU of CVV or PBS diluent. Dams were monitored and weighed daily and retro-orbitally bled on 4 DPI, followed by euthanasia and necropsies on 5 DPI. Maternal tissues (brain, liver, spleen, placenta, kidney, heart, and lung) and fetus heads were harvested from each dam, weighed, and stored in media until assayed for virus. Tissues were assayed for virus as described above.

Histopathology and organ loads in harvested tissues

Harvested tissues were homogenized using a Qiagen TissueLyser (Qiagen, Germantown, MD) for 5 min at 26,000 Hz and clarified by centrifugation for 5 min at 5510 × g. Samples were then titrated by plaque assays on Vero-76 cells to estimate organ virus loads as above. Virus groups were blinded, tissues were formalin fixed, paraffin embedded, sectioned, and hematoxylin and eosin (H&E) stained by the ViTALS diagnostic lab (Virginia Tech, Blacksburg, VA, USA). Pathology was scored blind by a board-certified veterinary pathologist. All tissues were graded individually for the presence and degree of cellular degeneration, cell death, and inflammation. Each was graded on a scale from 0 to 3 where 0 = no lesions observed, 1 = mild lesions observed, 2 = moderate lesions observed, and 3 = severe lesions observed. Individual scores for each parameter were then summed for each tissue for a total histopathology score for each organ.

Hemogram sysmex and Immunoassay in IFN-αβR$^{-/-}$ mice

Three-month old mice were separated into three groups, including CVV 4B ($n = 15$), CVV W08491 ($n = 15$), and PBS ($n = 15$). Mice were challenged with 10^4 PFU of CVV or PBS diluent. On 1, 3, and 5 DPI, five mice from each group were euthanized and cardiac bleeds performed. Whole blood (300 µl) was placed into EDTA microtainer tubes and mixed thoroughly to avoid clotting. Samples were sent to the ViTALS Animal Laboratory (Virginia Tech) and a full hemogram panel performed. Sera ($n = 12$ per group) collected on 1, 3 and 5 DPI were analysed using a FirePlex 96-Key Cytokines Mouse Immunoassay for 17 cytokines/chemokines (Abcam, Cambridge, MA). Samples were run in duplicate at a 1:2 dilution.

CVV pathogenesis in immune-competent CD-1 suckling mice

A CD-1 suckling mouse model was used to assess CVV neurovirulence, potential differences in strain pathogenicity, and compare the pathogenicity of these strains to strains used in previous studies. Six E17 pregnant dams were purchased from Charles River Laboratories (Strain: CD-1® IGS; Wilmington, MA, USA). Dams were equally divided into three groups CVV 4B ($n = 2$), CVV W08491 ($n = 2$) and PBS ($n = 2$; unchallenged healthy controls) and allowed to acclimatize and birth pups. Two days after birth, pups were intracranially inoculated with ~10^4 PFU of respective virus, or PBS diluent. Suckling mice were weighed and monitored daily for 14 DPI for signs of disease. On 3 and 5 DPI, brains were harvested from 3 mice per group for histopathology and virus quantification to assess replication kinetics. Upon visual manifestation of disease (cyanosis, unresponsiveness) or on collection days, mice were euthanized.

CVV pathogenesis in seven-week-old immune-competent C57BL/6J mice

C57BL/6J mice were obtained from The Jackson Laboratory (Strain: B6; Bar Harbor, ME, USA) and separated into five groups CVV 4B + MAR1-5A3 ($n = 8$), CVV 4B + Isotype IgG_1 Control ($n = 8$), CVV W08491+ MAR1-5A3 ($n = 8$) CVV W08491+ Isotype

IgG_1 Control ($n = 8$), and PBS ($n = 8$; unchallenged healthy controls). One day prior to inoculation, mice were administered 2.5 mg of either MAR1-5A3, Isotype IgG_1 Control (Leinco Technologies; St. Louis, MO, USA) or PBS intraperitoneally. Mice were then subcutaneously inoculated with 10^4 PFU of CVV or PBS diluent. Following infection, mice were given two additional 1 mg doses of MAR1-5A3, Isotype IgG_1, or PBS on 1 and 4 DPI. Mice were bled retro-orbitally daily for 4 DPI to assess viremia. After challenge, mice were monitored for weight changes, signs of disease, and mortality for 14 days.

Statistical analysis

Data normality was assessed using a combination of Q-Q plot and box-plot analyses. One-way ANOVAs and mixed-effects analyses followed by a Dunnett's multiple comparison test, and Logrank Mantel–Cox tests were performed using GraphPad Prism version 9.1.0.

Results

CVV infection results in significant morbidity and mortality in immune-compromised IFN-αβR$^{-/-}$ mice

To explore CVV pathogenesis and potential strain-specific differences in murine pathogenesis, 3- and 6-week-old IFN-αβR$^{-/-}$ mice were subcutaneously inoculated with CVV or PBS diluent. Six-week-old mice began showing signs of illness on 5 DPI and symptoms worsened until euthanasia. Signs of disease included lethargy, disorientation, hunched posture, and sunken eyes, eventually leading to paralysis and unresponsiveness. Mice in the W08491 group succumbed to illness by 8 DPI. However, the 4B group demonstrated 100% mortality by 12 DPI (Figure 1(a)). Mice from both infectious groups showed rapid weight loss starting 5 DPI (Figure 1(b)). Both groups presented significant viremia, achieving titers as high as 8.01 $\log_{10}$ PFU/ml for 4B and 6.53 $\log_{10}$ PFU/ml for W08491 4 DPI (Figure 1(c)). CVV was detected in all tissues, with the liver and spleens showing the greatest viral loads. Similarly, histopathologic examination of liver and spleen showed that lesions were present in both 3-week and 6-week-old CVV-infected mice. At all time points, histopathologic scores for the spleen and liver of CVV-infected mice were higher than the PBS groups (data not shown).

Both viruses caused significant and rapid mortality in 3-week-old mice, with 100% mortality by 5 DPI in the 4B group, and 6 DPI for the W08491 group (Figure 2(a)). Rapid weight loss was observed in both CVV groups starting on 3 DPI until euthanasia (Figure 2(b)). Viremia was detected at high levels, with both viruses presenting comparable titers on all days except on day 1 post infection, where viremia was only detected in the W08491 group ($n = 1$) (Figure 2(c)).

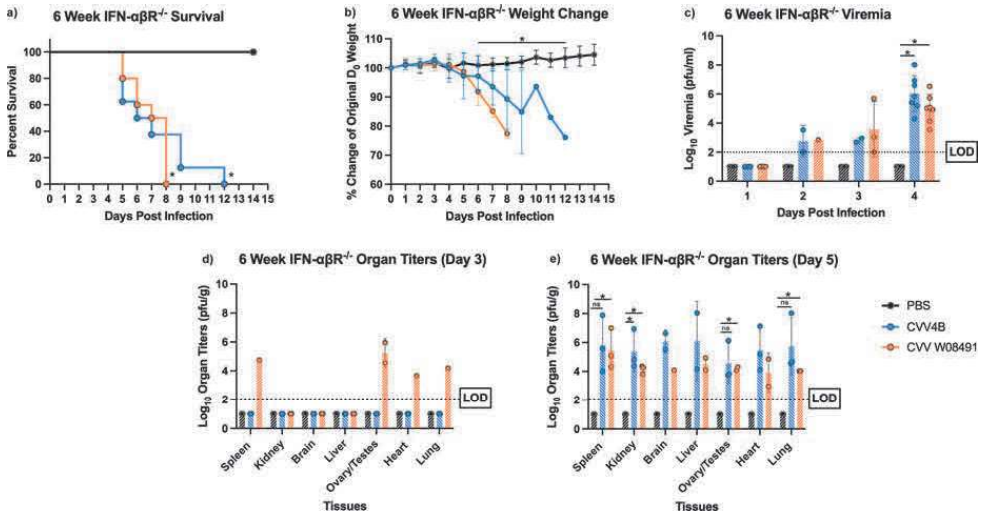


Figure 1. Cache Valley Virus (CVV) infection results in significant viremia, morbidity, and mortality in 6-week-old IFN-αβR$^{-/-}$ mice. Six-week-old mice (CVV 4B $n = 14$, CVV W08491 $n = 14$, PBS $n = 13$) were challenged subcutaneously with 10^4 plaque forming units (PFU) of virus, and (a) survival and (b) weight change measured daily for 14 days post infection (DPI). (c) Viremia was measured on 1–4 DPI, and organ titers for spleen, kidney, brain, liver, ovary/testes, heart and lung were measured on (d) 3 DPI and (e) 5 DPI. Each data point plotted represents the mean values and error bars indicate standard deviation. The limit of detection (LOD) is indicated with a dotted line. Statistical significance among groups were analysed by log-rank (Mantel-Cox) test in (a), and a mixed effects analysis with a Dunnett's multiple comparison test in (b–e). Statistically significant values are denoted by * ($p < 0.05$).

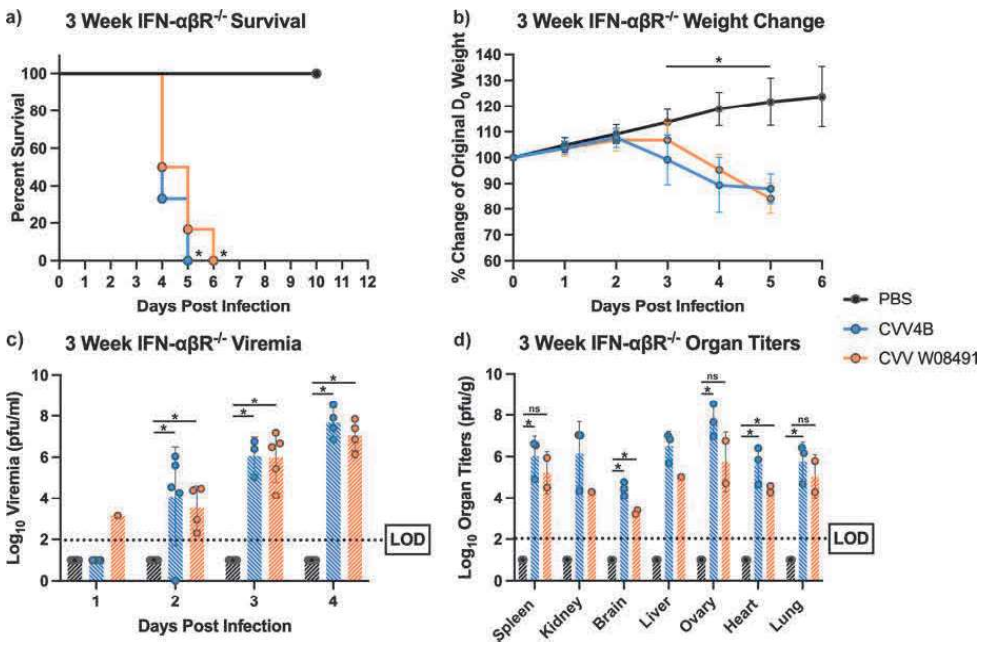


Figure 2. Cache Valley Virus (CVV) infection results in significant morbidity and mortality in 3-week-old IFN-αβR$^{-/-}$ mice. Three-week-old mice ($n = 9$/group) were challenged subcutaneously with 10^4 plaque forming units (PFU) of virus and (a) survival and (b) weight change measured daily for 10 days post infection (DPI). (c) Viremia was measured on 1–4 d post infection, and (d) organ titers measured for spleen, kidney, brain, liver, ovary, heart and lung harvested 3 DPI. Each data point plotted represents the mean values and error bars indicate standard deviation. The limit of detection (LOD) is indicated with a dotted line. Statistical significance among groups were analysed by log-rank (Mantel-Cox) test in (a), and a mixed effects analysis with a Dunnett's multiple comparison test in (b–d). Statistically significant values are denoted by * ($p < 0.05$).

Both virus groups showed high organ titer loads, with spleen, liver and ovaries presenting with the highest viral loads (Figure 2(d)). The 4B group showed the highest organ titers across all tissues ($p < 0.05$). This is supported by the histopathology that showed the highest lesion scores for the liver and spleen were encountered in the 4B groups (data not shown). No significant microscopic lesions were observed in the kidney, lung, or reproductive organs of CVV-infected mice when compared to the PBS group.

CVV infection and tissue tropism in 1-year-old IFN-αβR$^{-/-}$ mice

To further dissect CVV's tissue tropism and assess age-dependence, we inoculated 1-year old IFN-αβR$^{-/-}$ mice subcutaneously. Weight loss was observed in both CVV groups starting 4 DPI (Figure 3(a)). Viremia was observed in both virus-infected groups on days 2–6 DPI (Figure 3(b)). However, for organ viral loads, only 4B ($n = 2$) showed low titers in various tissues on day 2 post infection (Figure 3(c)), including the spleen, kidney, heart and lungs. By 4 DPI, both 4B and W08491 showed significant virus loads in all tissues with the exception of the brain, where no virus was detected in the W08491 challenged mice (Figure 3(d)). At 6 DPI, all organs demonstrated higher virus titers, with the highest titers observed in the liver, spleen, kidney, and reproductive tissues. The lowest titers were observed in the brain (Figure 3(e)). Similar trends were observed with histological samples. CVV-infected groups began to show lesions in the liver by 2 DPI while lesions in the spleen were not observed until 4 DPI. For both organs, lesions became more severe with time and were the most significant by 6 DPI (Figure 4). Minimal, if any, lesions were observed in the PBS groups. No significant microscopic lesions were observed in the kidney, lung, or reproductive organs of CVV-infected mice when compared to the PBS group.

In utero CVV transmission causes severe morbidity and mortality in pregnant IFN-αβR$^{-/-}$ dams and fetuses

To further explore CVV's teratogenicity phenotype, we explored *in utero* transmission of CVV and its effect on fetal development. We inoculated pregnant IFN-αβR$^{-/-}$ dams subcutaneously with a respective CVV strain or PBS diluent 10.5 days after the onset of embryonic development (E10.5). Pregnant dams in the W08491 group showed weight loss starting 3,

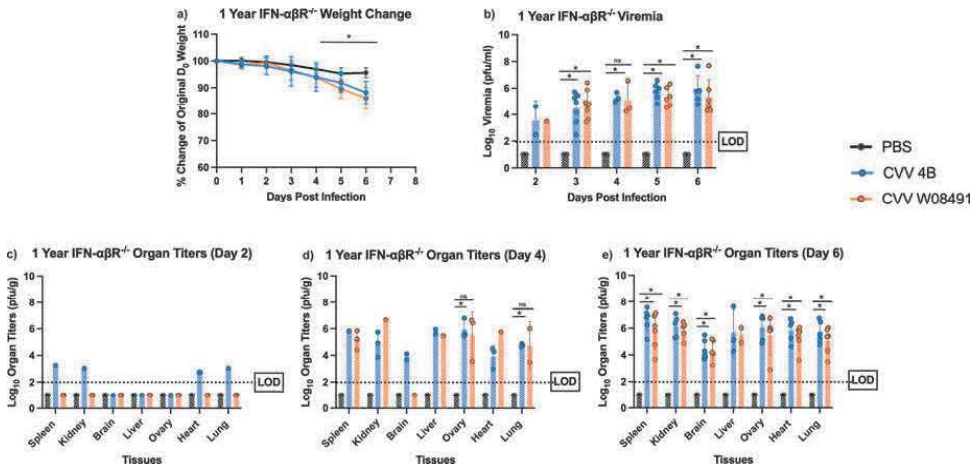


Figure 3. Cache Valley Virus (CVV) infection results in significant viremia, morbidity, and mortality in 1-year-old IFN-αβR$^{-/-}$ mice. One-year-old mice (CVV 4B $n = 12$, CVV W08491 $n = 12$, PBS $n = 8$) were challenged subcutaneously with 10^4 plaque forming units (PFU) of virus and (a) weight change was measured daily for 6 days post infection (DPI). (b) Viremia was measured on 2–6 DPI, and organ titers for spleen, kidney, brain, liver, ovary, heart and lung at (c) 2 DPI, (d) 4 DPI, and (e) 6 DPI. Each data point plotted represents mean values, and error bars indicate standard deviation. The limit of detection (LOD) is indicated with a dotted line. Statistical significance among groups were analysed by a mixed effects analysis with a Dunnett's multiple comparison test in (a-e). Statistically significant values are denoted by * ($p < 0.05$).

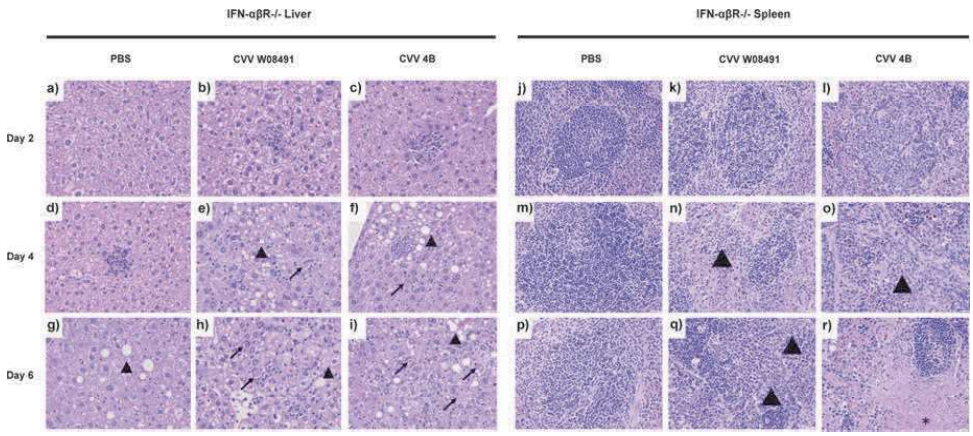


Figure 4. Cache Valley Virus (CVV) infection shows histological effects in livers and spleens of 1-year-old IFN-αβR$^{-/-}$ mice. H&E-stained micrographs of liver (a–i) and spleen (j–r). Liver sections were evaluated for evidence of hepatocyte degeneration characterized by vacuolation of the cytoplasm (arrowhead); cell death characterized by cell swelling, hypereosinophilia of the cytoplasm, and fragmentation of the nucleus (arrow); and degree of inflammation which was predominantly composed of neutrophils and variable numbers of macrophages. Spleen sections were evaluated for inflammation which was predominantly composed of neutrophils (arrowheads) as well as evidence of cell death characterized by necrosis and cellular debris (asterisk). Images were captured at 40x magnification.

and 4 DPI in the 4B group (Figure 5(a)). Viremia was observed in all CVV-infected groups (Figure 5(b)) confirming infection. High viral loads were detected in all maternal tissues taken at necropsy, 5 DPI (Figure 5(c)). Interestingly, there were statistically significant differences in weights between CVV infected neonates and PBS controls (Figure 5(d)). High viral loads were observed in the placentas of infected dams and associated brains of their neonates. Virus titers were nearly 10,000-fold higher in the placentas when compared to the fetus brains (Figure 5(e)) and 100-fold higher when compared to viremia during necropsy. *In utero* transmission rates to neonates were as high as 60% for W08491- and 46% for 4B-infected mice.

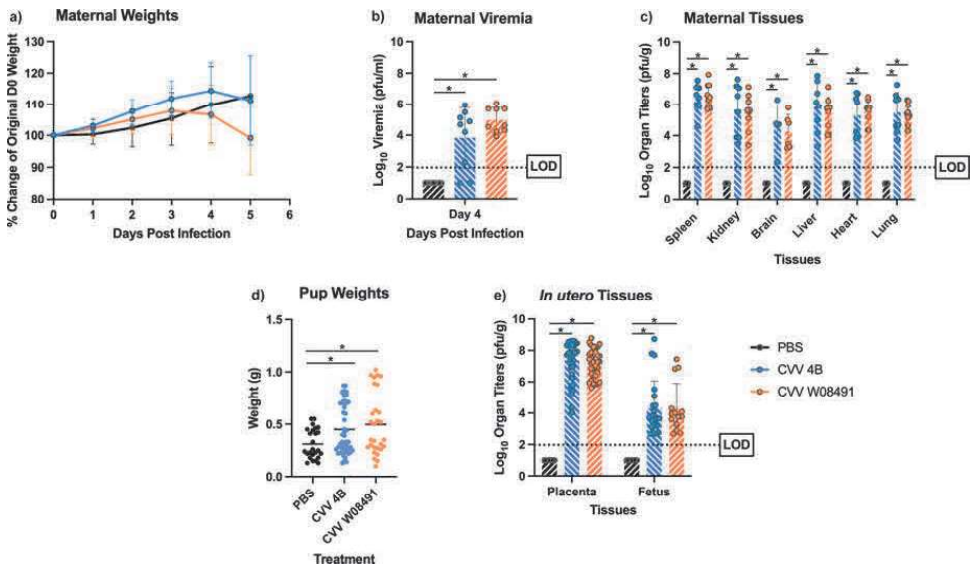


Figure 5. Cache Valley Virus (CVV) infection of IFN-αβR$^{-/-}$ mice results in high in *utero* transmission rates and congenital abnormalities. Pregnant IFN-αβR$^{-/-}$ dams (CVV 4B $n = 8$, CVV W08491 $n = 9$, PBS $n = 7$) were challenged subcutaneously with 10^4 plaque forming units (PFU) of virus and (a) weight change measured daily for 5 days post infection (DPI). (b) Viremia was measured on 4 DPI. (c) Maternal organ titers for spleen, kidney, brain, liver, ovary, heart and lung, (d) pup weights, and (e) *in utero* tissues, including placenta and fetus brains were assayed for virus titers by plaque assays on Vero-76 cells. Each data point plotted represents the mean values and error bars indicate standard deviation. The limit of detection (LOD) is indicated with a dotted line. Statistical significance among groups were analysed by a mixed effects analysis with a Dunnett's multiple comparison test in (a,c,e), one-way ANOVA with Dunnett's multiple comparison test in (b,d), Statistically significant values are denoted by * ($p < 0.05$).

CVV infection causes thrombocytopenia, lymphocytopenia and dysregulated cytokine responses in IFN-αβR$^{-/-}$ mice

To examine hematological effects of CVV, IFN-αβR$^{-/-}$ mice were challenged with CVV or PBS diluent. CVV 4B infected mice showed statistically significant differences in white blood cells (WBC), eosinophils, lymphocytes and platelets in comparison to PBS controls (Table S2). No differences were observed between the CVV W08491 and the PBS controls. None of the remaining 12 measurements taken showed significant differences between virus infected and PBS control mice. Thrombocytopenia and lymphocytopenia were pronounced at 5 DPI in the W08491 group. CVV 4B and W08491 showed statistically significant differences in CXCL1, GM-CSF, IFN-γ, IL-10, IL-17A, IL-4, IL-5, IL-9, MCP1, MIP1α, and MIP1β when compared to PBS controls (Table S4).

CVV replicates efficiently and is neurovirulent in immune-competent suckling mice

We investigated CVV neurovirulence by intracranially inoculating 2-day old CD-1 suckling mice. Mice were inoculated with 10^4 PFU of CVV 4B ($n = 28$), CVV W08491 ($n = 23$) or PBS ($n = 15$). By 4 DPI, all of the mice in the CVV 4B group had to be euthanized due to severe disease (i.e. cyanotic or unresponsive) (Figure 6(a)). All mice steadily gained weight until 3 DPI (Figure 6(b)), after which CVV 4B-infected mice began to show symptoms of disease and required euthanasia. The CVV W08491 mice began to show symptoms at 7 DPI, and mice were euthanized on 8 DPI due to severe disease (hypoxia, lethargy, moribund). Brain samples harvested from mice ($n = 3$) on 3 and 5 DPI to evaluate organ load titers showed high titers peaking at 9.52 $\log_{10}$ PFU/g for CVV 4B and 8.13 $\log_{10}$ PFU/g for CVV W08491 infected mice (Figure 6(c)). Histological analysis of brain from CVV-infected mice showed evidence of white matter vacuolation, cell death, and inflammation characterized predominantly by neutrophils (Figure 6(d–i)). Histopathological scores for tissues analysed in all IFN-αβR$^{-/-}$ studies are shown in Table S3.

CVV infection causes reduced disease in C57Bl/6J mice

To explore CVV pathogenesis in an immune-competent mouse model, we subcutaneously inoculated C57BL/6J mice following an isotype or anti-IFN antibody blockade. Mice were administered MAR1-5A3, Isotype IgG1 control or PBS as described in the

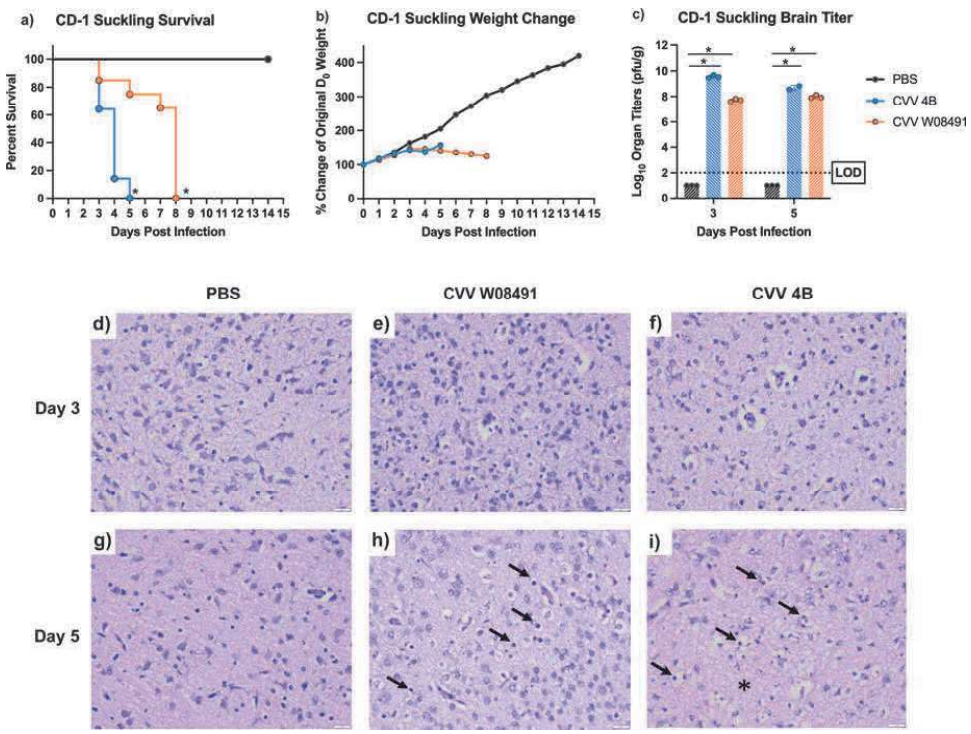


Figure 6. Cache Valley Virus (CVV) infection produces high viral loads and is lethal in CD-1 suckling mice. Two-day-old suckling mice (CVV 4B $n = 28$, CVV W08491 $n = 23$, PBS $n = 15$) were challenged intracranially with 10^4 plaque forming units (PFU) and (a) survival and (b) weight change measured daily for 14 days post infection (DPI). (c) Virus titers were measured in brain sections harvested on 3 and 5 DPI. Each data point plotted represents the mean values, and error bars indicate standard deviation. The limit of detection (LOD) is indicated with a dotted line. Statistical significance among groups was analysed by logrank (Mantel-Cox) test in (a) a mixed effects analysis with a Dunnett's multiple comparison test in (b,c) Statistical significance is denoted by * ($p < 0.05$). Brain sections were analysed for histopathological effects after H&E staining 3 and 5 DPI. Sections were evaluated for degree of necrosis characterized by neuropil hyalinization and vacuolation (asterisk) as well as shrinkage of cells and condensation of nuclei (arrows). Sections were also evaluated for degree of inflammation as well which was minimal and mostly characterized by rare infiltration by neutrophils (not shown), on (d,e,f) 3 DPI and (g,h,i) 5 DPI. Images were captured at 40x magnification.

methods. All mice survived the 14-day study except for one female in the CVV 4B MAR1-5A3 group which had to be euthanized on 9 DPI. The CVV 4B mice administered MAR1-5A3 showed moderate weight loss (Figure S1), but no disease was observed among any of the virus-infected groups throughout this study. No viremia was detected in this model 1– 4 DPI (data not shown).

Discussion

CVV is a prevalent emerging pathogen of significant importance to agricultural and human health in North America. Emergence in livestock can result in severe agroeconomic losses due to severe embryonic lethality associated with infection during pregnancy. Although CVV pathogenesis has been well described in ruminants [1,25,32,34], small animal models are still unavailable, which limits our ability to study CVV pathogenesis and perform preclinical testing of therapeutics. Thus, we explored various immune -competent and -compromised murine models using two CVV strains from both phylogenetic lineages.

Herein we report the development of a variety of novel CVV infection and disease murine models. Results from our CD-1 suckling mouse model show comparable mortality to previous studies [4,35]. Previously, CVV was isolated and/or amplified by intracranial inoculation of suckling mice, and mortality was observed in mice 6–13 DPI, although faster mortality rates were observed with mouse-adapted strains. Our model showed exceptional sensitivity and was able to distinguish differences in neurovirulence between both CVV strains studied here. This model should therefore be preferentially employed when comparing neurovirulence among strains. The IFN-$\alpha\beta R^{-/-}$ models used presented with significant disease and lethal infections, with minimal differences in age-dependent pathogenesis. The IFN-$\alpha\beta R^{-/-}$ model

is an especially susceptible model for arbovirus infections and has been widely employed for pathogenesis and therapeutic testing [36]. This model is therefore appropriate for short- or long-term prophylactic and therapeutic studies. CVV infection of IFN-αβR$^{-/-}$ mice resulted in pronounced thrombocytopenia and lymphopenia, as well as viral infection derived characteristics such as depleted WBCs and nucleated red blood cells (NRBC). NRBCs are generally low in healthy mice but can be increased or severely decreased in pathologic states of any kind. Our progressive timepoints (days 1, 3, 5) showed a decrease which may be associated with the histopathologic inflammation observed. This has previously been recorded in human infections with CVV and other bunyavirus infections [16–19], which provides further support of an accurate reflection of CVV disease in this model. The immunoassays showed the most significant cytokine/chemokine responses in CVV-infected IFN-αβR$^{-/-}$ mice at 3 and 5 DPI. These data suggest that CVV elicits significant pro- and anti-inflammatory cytokine responses, and also strong support for CVV activation of signalling for recruitment of inflammatory cells.

The *in utero* transmission study shows high rates of transmission to neonates. Particularly noteworthy is the fact that IFN-αβR$^{-/-}$ dams experienced spontaneous abortions 5 days after challenge. This abortive effect is a characteristic of CVV in ruminants [1,26,32]. The significant viral titers estimated from placental tissues suggest that CVV is particularly fit for replication in these tissues. This increased replication likely resulted in the large virus burden detected in neonatal brains. CVV infected neonates show statistically significant increased weights in comparison to control neonates; which might suggest congenital abnormalities such as macrocephaly, hydranencephaly, or other developmental defects in infected neonates (Figure 5(d)). Macrocephaly was previously observed in human newborns infected with CVV [23]. These studies confirm observational studies related to CVV outbreaks over the past 40 years, demonstrating severe neurological and physical deformities in ruminant fetuses and humans [21,23,25,32]. Although arthrogryposis (i.e. congenital joint contracture that results in curving of joints) is another common symptom in neonates infected with CVV [26,32,37,38], we did not assess musculoskeletal defects of CVV-infected IFN-αβR$^{-/-}$ neonates. This should be further explored in this model. Further studies exploring the effects of CVV infections at different gestational periods in IFN-αβR$^{-/-}$ mice are still needed, as previously done in sheep [32]. Overall, this model can be a useful tool for understanding the mechanisms underlying the induction of spontaneous abortions, *in utero* transmission and assessing therapeutic efficacy.

C57BL/6J mice are resistant to CVV infection, as seen previously with outbred mice [39,40], likely due to an inability of CVV to antagonize murine innate immune responses. Despite administering a large type I interferon blockade, C57BL/6J mice did not present with viremia or significant mortality, commonly observed with IFN-αβR$^{-/-}$ mice. However, it must be noted that the type I interferon blockade was not evaluated for its efficacy in this study. Weight loss was the only disease signal observed in this model. Further studies are needed to determine if different challenge doses, additional innate immune response knockouts, or different interferon blockade regimens can increase disease in this model. Additionally, future studies using knockout mice such as STAT1$^{-/-}$ and STAT2$^{-/-}$ that are susceptible to orthobunyavirus infection [40] are also needed to identify which innate response pathways restrict CVV replication in mice.

Although CVV replicates efficiently when administered directly to the brain, our data shows lower titers when comparing brain samples to other organs when inoculated peripherally. Our studies show that CVV replicated to the highest titers in spleens, livers, ovaries, and placentas after peripheral inoculation. This suggests CVV has a wide tissue tropism and may be viserotropic. Future studies are needed to determine if different infection routes play a role in CVV tissue tropism.

Recent studies suggest that there has been a lineage replacement event in the northeastern U.S.A., and that lineage II strains now predominate the north eastern U.S. [8]. Despite only using 2 strains, our studies suggest that 4B (lineage II) may be more neurovirulent than W08491 (lineage I). Altogether, our studies show limited differences in disease among strains, with the exception of increased viral loads and neurovirulence with 4B. Further studies that include larger numbers of strains from both phylogenetic lineages are needed to rigorously determine if altered pathogenesis among strains could have influenced the lineage displacement in the northeastern U.S.A.

In conclusion, our results indicate that IFN-αβR$^{-/-}$ mice are a useful tool for studying CVV pathogenesis and *in utero* transmission and can help inform therapeutic and vaccine testing. Immune-competent mice are generally resistant to infection and only show disease in an age dependent manner. Our studies have provided valuable details on important characteristics of CVV infection, pathogenesis, and immune responses in relevant and commercially available mouse models. With seropositivity rates in parts of the eastern USA as high as 96.4% in sheep [5,15], and the continuing geographic expansion of mosquitoes capable of transmitting the pathogen, like *Ae. aegypti* and *Ae. albopictus* [9], the risk of emergence for CVV is high, and interventions are urgently needed.

Acknowledgements

We thank Dr. Philip Armstrong for sharing cache valley virus isolates. We thank the Fralin Life Science Institute for administrative and technical support. We would also like to acknowledge Virginia Tech's open access subvention fund for providing financial support for publication fees.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by grants from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under Award Numbers K22AI125474 and R01AI153433 to AJA. This work was also supported by a seed grant from the Center for Emerging, Zoonotic, and Arthropod-borne Pathogens at Virginia Tech. KL is supported by an Institute for Critical Technologies and Applied Science Doctoral fellowship, Virginia Tech. MT is supported by a George Washington Carver fellowship from the College of Agriculture and Life Sciences, Virginia Tech. JAM was funded through a National Science Foundation Fellowship under award number DGE-1545261.

ORCID

Albert J. Auguste http://orcid.org/0000-0001-9048-0041

References

[1] Edwards JF. Cache Valley virus. Vet Clin North Am Food Anim Pract. 1994;10(3):515–524.
[2] Calisher CH, Francy DB, Smith GC, et al. Distribution of Bunyamwera serogroup viruses in North America, 1956–1984. Am J Trop Med Hyg. 1986 Mar;35 (2):429–443.
[3] Noronha LE, Wilson WC. Comparison of two zoonotic viruses from the order Bunyavirales. Curr Opin Virol. 2017 Dec;27:36–41.
[4] Holden P, Hess AD. Cache Valley virus, a previously undescribed mosquito-borne agent. Science. 1959 Oct 30;130(3383):1187–1188.
[5] Uehlinger FD, Wilkins W, Godson DL, et al. Seroprevalence of Cache Valley virus and related viruses in sheep and other livestock from Saskatchewan, Canada. Can Vet J. 2018 Apr;59 (4):413–418.
[6] Cornell University CoVM. Cache Valley Virus. 2021.
[7] Brockus CL, Grimstad PR. Sequence analysis of the medium (M) segment of Cache Valley virus,1 with comparison to other Bunyaviridae. Virus Genes. 1999;19(1):73–83.
[8] Armstrong PM, Andreadis TG, Anderson JF. Emergence of a new lineage of Cache Valley virus (Bunyaviridae: Orthobunyavirus) in the Northeastern United States. Am J Trop Med Hyg. 2015 Jul;93(1):11–17.
[9] Waddell L, Pachal N, Mascarenhas M, et al. Cache Valley virus: A scoping review of the global evidence. Zoonoses Public Health. 2019 Nov;66(7):739–758.
[10] McLean RG, Calisher CH, Parham GL. Isolation of Cache Valley virus and detection of antibody for selected arboviruses in Michigan horses in 1980. Am J Vet Res. 1987 Jul;48(7):1039–1041.
[11] Kokernot RH, Hayes J, Tempelis CH, et al. Arbovirus studies in the Ohio-Mississippi Basin, 1964–1967. IV. Cache Valley virus. Am J Trop Med Hyg. 1969 Sep;18 (5):768–773.
[12] Hoff GL, Spalatin J, Trainer DO, et al. Isolation of a bunyamwera group arbovirus from a naturally infected caribou. J Wildl Dis. 1970 Oct;6 (4):483–487.
[13] Blackmore CG, Grimstad PR. Cache Valley and Potosi viruses (Bunyaviridae) in white-tailed deer (Odocoileus virginianus): experimental infections and antibody prevalence in natural populations. Am J Trop Med Hyg. 1998 Nov;59(5):704–709.
[14] Neitzel DF, Grimstad PR. Serological evidence of California group and Cache Valley virus infection in Minnesota white-tailed deer. J Wildl Dis. 1991 Apr;27(2):230–237.
[15] Meyers MT, Bahnson CS, Hanlon M, et al. Management factors associated with operation-level prevalence of antibodies to Cache Valley virus and other Bunyamwera serogroup viruses in sheep in the United States. Vector Borne Zoonotic Dis. 2015 Nov;15(11):683–693.
[16] Baker M, Hughes HR, Naqvi SH, et al. Reassortant Cache Valley virus associated with acute febrile, non-neurologic illness, Missouri. Clin Infect Dis. 2021 Feb 25;ciab175.
[17] Nguyen NL, Zhao G, Hull R, et al. Cache valley virus in a patient diagnosed with aseptic meningitis. J Clin Microbiol. 2013 Jun;51(6):1966–1969.
[18] Sexton DJ, Rollin PE, Breitschwerdt EB, et al. Life-Threatening Cache Valley virus infection. N Engl J Med. 1997;336(8):547–549.
[19] Campbell GL, Mataczynski JD, Reisdorf ES, et al. Second human case of Cache Valley virus disease. Emerging Infect Dis.. 2006;12(5):854–856.
[20] Andreadis TG, Armstrong PM, Anderson JF, et al. Spatial-temporal analysis of Cache Valley virus (Bunyaviridae: Orthobunyavirus) infection in anopheline and culicine mosquitoes (diptera: culicidae) in the Northeastern United States, 1997–2012. Vector Borne Zoonotic Dis. 2014 Oct;14(10):763–773.
[21] Buescher EL, Byrne RJ, Clarke GC, et al. Cache Valley virus in the Del Mar Va Peninsula. I. Virologic and serologic evidence of infection. Am J Trop Med Hyg. 1970 May;19(3):493–502.
[22] Work TH. Serological evidence of Arbovirus infection in the seminole Indians of Southern Florida. Science. 1964 Jul 17;145(3629):270–272.
[23] Calisher CH, Sever JL. Are North American Bunyamwera serogroup viruses etiologic agents of human congenital defects of the central nervous system? Emerg Infect Dis. 1995 Oct-Dec;1(4):147–151.
[24] Wilson MR, Suan D, Duggins A, et al. A novel cause of chronic viral meningoencephalitis: Cache Valley virus. Ann Neurol. 2017 Jul;82(1):105–114.
[25] Chung S, Livingston CW, Edwards JF, et al. Evidence that Cache Valley virus induces congenital malformations in sheep. Vet Microbiol. 1990 Feb;21 (4):297–307.
[26] Edwards JF, Livingston CW, Chung SI, et al. Ovine arthrogryposis and central nervous system malformations associated with in utero Cache Valley virus infection: spontaneous disease. Vet Pathol. 1989 Jan;26(1):33–39.

[27] Hayles LB, Lversen JO. Cache Valley virus: experimental infection inCuliseta inornata. Can J Microbiol. 1980 Mar;26(3):287–290.
[28] Yang F, Chan K, Marek PE, et al. Cache Valley virus inAedes japonicus japonicusMosquitoes, Appalachian region, United States. Emerg Infect Dis. 2018 Mar;24 (3):553–557.
[29] Ayers VB, Huang Y-S, Lyons AC, et al. Infection and transmission of Cache Valley virus by Aedes albopictus and Aedes aegypti mosquitoes. Parasit Vectors. 2019 Jul 31;12(1):384.
[30] Shan C, Xie X, Shi P-Y. Reverse genetics of zika virus. Methods Mol Biol. 2017;1602:47–58.
[31] Auguste AJ, Kaelber JT, Fokam EB, et al. A newly isolated Reovirus has the simplest genomic and structural organization of any Reovirus. J Virol. 2015 Jan;89 (1):676–687.
[32] Chung SI, Livingston CW, Jr, Edwards JF, et al. Congenital malformations in sheep resulting from in utero inoculation of Cache Valley virus. Am J Vet Res. 1990 Oct;51(10):1645–1648.
[33] Wilson SN, Lopez K, Coutermash-Ott S, et al. La Crosse virus shows strain-specific differences in pathogenesis. Pathogens. 2021 Mar 29;10(4):400.
[34] Crandell RA, Livingston CW, Jr, Shelton MJ. Laboratory investigation of a naturally occurring outbreak of arthrogryposis-hydranencephaly in Texas sheep. J Vet Diagn Invest. 1989 Jan;1(1):62–65.
[35] McConnell S, Livingston C, Jr, Calisher CH, et al. Isolations of Cache Valley virus in Texas, 1981. Vet Microbiol. 1987 Jan;13(1):11–18.
[36] Marin-Lopez A, Calvo-Pinilla E, Moreno S, et al. Modeling arboviral infection in mice lacking the interferon alpha/beta receptor. Viruses. 2019 Jan 8;11(1):35.
[37] Sodergard J, Hakamies-Blomqvist L, Sainio K, et al. Arthrogryposis multiplex congenita: perinatal and electromyographic findings, disability, and psychosocial outcome. J Pediatr Orthop B. 1997 Jul;6(3):167–171.
[38] Hall JG. Arthrogryposis multiplex congenita: etiology, genetics, classification, diagnostic approach, and general aspects. J Pediatr Orthop B. 1997 Jul;6(3):159–166.
[39] Habjan M, Pichlmair A, Elliott RM, et al. NSs protein of rift valley fever virus induces the specific degradation of the double-stranded RNA-dependent protein kinase. J Virol. 2009 May;83(9):4365–4375.
[40] Reynolds ES, Hart CE, Hermance ME, et al. An overview of animal models for arthropod-borne viruses. Comp Med. 2017 Jun 1;67(3):232–241.

Supplemental Materials

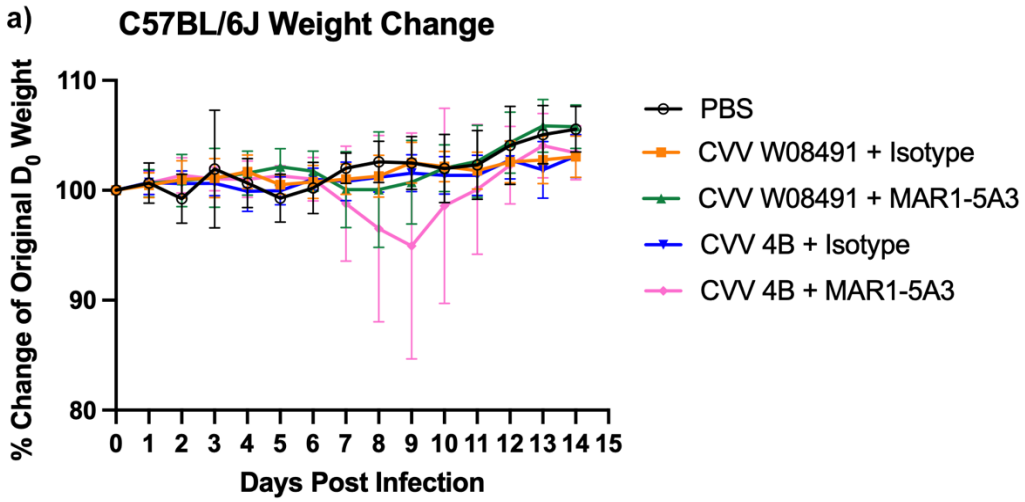


Figure S1. CVV does not present with severe disease in C57BL/6J mice. Seven-week-old mice (*n*=8/group) were administered 250µg of either MAR1-5A3, or Isotype IgG_1 Control one day prior to inoculation, and the following day inoculated subcutaneously with 10^4 plaque forming units (PFU) of virus. Following infection, mice were given two additional 100µg doses of MAR1-5A3, Isotype IgG_1 and PBS, on 1- and 4-days post infection (DPI). Weight change was measured daily for 14 DPI. No viremia was detected in these mice throughout the study period. Each data point plotted represents the mean values and error bars indicate standard deviation. The limit of detection (LOD) is indicated with a dotted line. Statistical significance among groups was analyzed by a mixed effects analysis with a Dunnett's multiple comparison test. Statistically significant values are denoted by * ($p<0.05$).

Table S1. Meta-data for viruses used in this study.

Strain Name	Source	Location of Isolation	Year of Isolation	Accession Number	Cell Type	Stock Titer (PFU/mL)	Viral Lineage
4B	*Aedes japonicus*	Blacksburg, VA	2015	MZ612419, MZ612420 MZ612423	Vero	7.70×10^7	2
					C6/36	1.33×10^5	
W08491	*Culex tarsalis*	North Dakota	2005	MZ612418, MZ612421 MZ612422	Vero	1.09×10^7	1
					C6/36	5.72×10^3	

Table S2. Cache Valley virus infected IFN-abR$^{-/-}$ mice demonstrated significant differences in hemogram measurements during infection.

Measurements	Day 1			Day 3			Day 5		
	Negative Control	CVV W08491	CVV 4B	Negative Control	CVV W08491	CVV 4B	Negative Control	CVV W08491	CVV 4B
RBC	9.316 ± 0.353	9.63 ± 0.184	9.353 ± 0.606	9.346 ± 0.503	9.726 ± 0.355	10.022 ± 0.349	9.530 ± 0.409	9.772 ± 0664	9.760 ± 0.372
Hemoglobin	15.700 ± 0.430	15.720 ± 0.259	15.775 ± 0.222	14.840 ± 0.971	15.520 ± 0.536	15.640 ± 0.422	15.500 ± 0.200	15.520 ± 0.455	15.500 ± 0.735
Hematocrit	49.080 ± 1.285	50.080 ± 0.968	48.725 ± 2.516	49.160 ± 1.385	50.060 ± 1.885	51.320 ± 1.782	51.180 ± 1.385	50.820 ± 2.752	50.750 ± 1.658
MCV	52.700 ± 0.735	52.020 ± 0.867	52.125 ± 1.024	52.620 ± 1.026	51.460 ± 0.207	51.220 ± 0.522	52.600 ± 0.418	52.600 ± 0.418	52.025 ± 0.574
MCHC	32.000 ± 0.510	31.400 ± 0.752	32.425 ± 1.253	30.180 ± 1.441	31.000 ± 0.640	30.520 ± 1.516	30.960 ± 1.539	30.620 ± 1.810	30.525 ± 0.645
NRBC Sysmex	13.600 ± 4.529	7.460 ± 1.379	16.325 ± 10.106	7.820 ± 3.273	9.300 ± 2.874	6.660 ± 4.442	9.380 ± 4.307	14.200 ± 8.997	6.000 ± 1.903
RDW-CV	15.800 ± 0.400	15.980 ± 0.455	15.500 ± 0.913	16.300 ± 0.200	16.540 ± 0.167	16.500 ± 0.167	16.380 ± 0.342	16.880 ± 0.492	16.100 ± 0.572
Retic %	4.080 ± 1.099	3.660 ± 0.428	3.700 ± 0.294	4.700 ± 1.913	3.800 ± 0.235	3.760 ± 0.261	3.820 ± 0.277	3.980 ± 0.377	3.850 ± 0.208
Reticulocyte #	376.640 ± 88.277	351.980 ± 39.109	342.525 ± 16.715	433.380 ± 148.445	369.340 ± 21.304	375.680 ± 31.821	363.680 ±29.170	389.300 ± 55.713	375.825 ± 26.638
WBC	8.342 ± 1.595	8.182 ± 0.952	9.155 ± 1.975	5.434 ± 1.506	4.298 ± 1.334	7.178 ± 1.512	9.592 ± 2.956	7.234 ± 1.137	5.125 ± 0.974*
Neutrophils	1.460 ± 0.699	0.900 ± 0.212	1.250 ± 0.332	0.780 ± 0.249	0.720 ± 0.130	1.060 ± 0.241	2.280 ± 1.221	3.040 ± 1.260	1.875 ± 0.330
Lymphocytes	5.960 ± 1.498	6.580 ± 1.038	6.975 ± 2.069	4.140 ± 1.390	3.220 ± 1.281	5.480 ± 1.305	6.060 ± 2.115	3.020 ± 1.375	2.100 ± 0.658*
Monocytes	0.620 ± 0.228	0.400 ± 0.071	0.550 ± 0.058	0.440 ±0.182	0.280 ± 0.084	0.500 ± 0.394	0.940 ± 0.550	0.900 ± 0.394	1.000 ± 0.216
Eosinophils	0.220 ± 0.045	0.220 ± 0.045	0.300 ± 0.000*	0.040 ± 0.055	0.060 ± 0.089	0.120 ± 0.045	0.240 ± 0.114	0.180 ± 0.084	0.100 ± 0.000
Basophils	0.080 ± 0.045	0.100 ± 0.000	0.100 ± 0.000	0.020 ± 0.045	0.000 ± 0.000	0.020 ± 0.045	0.060 ± 0.055	0.080 ± 0.045	0.075 ± 0.050
Platelets	1142.800 ± 281.557	1287.600 ± 52.662	1308.500 ± 28.219	1314.400 ± 239.755	1428.400 ± 104.820	1096.600 ± 672.587	1473.200 ± 148.571	1175.400 ± 305.062	916.750 ± 202.253**
MPV	7.080 ± 0.526	6.600 ± 0.071	6.775 ± 0.275	6.800 ± 0.141	7.000 ± 0.141	7.060 ± 0.709	7.060 ± 0.329	7.120 ± 0.356	7.000 ± 0.383

Each data point represents the mean and standard deviations. Statistically significant values are denoted by * ($p<0.05$).

Red Blood Cells (RBC), Hemoglobin, Hematocrit, Mean corpuscular volume (MCV), Mean corpuscular hemoglobin volume (MCHC), Nucleated Red Blood Cell Sysmex (NRBC Sysmex), Red blood cell distribution width (RDW-CV), Reticulocytes percent (Retic %), Reticulocyte number, White Blood Cells (WBC), Neutrophil, Lymphocytes, Monocytes, Eosinophils

Table S3. Cache Valley virus infected IFN-abR$^{-/-}$ mice demonstrated significant histopathology across tissues during infection.

Each data point was graded on a scale from 0-3 where 0=no lesions observed, 1=mild lesions observed, 2=moderate lesions observed, and 3=severe lesions observed. Individual scores for each parameter were then summed for each tissue for a total histopathology score for each org

Table S4. Cache valley virus infected IFN-abR$^{-/-}$ mice demonstrated significant differences in cytokine and chemokine responses during infection.

Parameters	Day 1			Day 3			Day 5		
	PBS	**CVV W08491**	**CVV 4B**	**PBS**	**CVV W08491**	**CVV 4B**	**PBS**	**CVV W08491**	**CVV 4B**
CXCL1	22.591 ± 8.013	18.341 ± 4.198	20.808 ± 2.715	17.811 ± 3.535	75.685 ± 71.821	86.150 ± 73.960	16.2525 ± 3.773	154.581 ± 170.972	190.609 ± 160.415*
GM-CSF	0.605 ± 0.641	0.200 ± 0.035	0.347 ± 0.222	0.312 ± 0.136	0.303 ± 0.088	0.225 ± 0.040	0.230 ± 0.049	0.298 ± 0.107	0.377 ± 0.098*
IFN-γ	0.689 ± 0.204	0.981 ± 0.671	0.430 ± 0.182*	2.720 ± 3.659	25.556 ± 19.620*	9.738 ± 11.521	0.755 ± 0.737	268.245 ± 305.249	269.594 ± 322.283
IL-1β	0.696 ± 0.430	0.299 ± 0.023	0.384 ± 0.128	0.354 ± 0.124	1.056 ± 1.117	0.383 ± 0.139	0.301 ± 0.054	0.514 ± 0.273	0.653 ± 0.294*
IL-10	5.160 ± 1.811	5.731 ± 1.878	4.798 ± 0.837	7.093 ± 1.819	13.255 ± 3.798*	7.506 ± 1.146*	4.343 ± 0.629	11.556 ± 5.797*	7.873 ± 5.299
IL-12p70	0.886 ± 0.531	1.338 ± 1.402	0.815 ± 0.443	0.815 ± 0.364	0.873 ± 0.537	0.645 ± 0.146	0.644 ± 0.194	3.191 ± 3.253	4.523 ± 4.094
IL-13	0.713 ± 0.549	0.308 ± 0.049	0.463 ± 0.244	0.515 ± 0.103	0.714 ± 0.233	0.618 ± 0.154	0.341 ± 0.083	0.796 ± 0.807	0.931 ± 0.653
IL-17A	1.318 ± 0.440	0.830 ± 0.323*	0.788 ± 0.322	0.485 ± 0.207	1.523 ± 0.510*	0.783 ± 0.278*	1.180 ± 0.839	0.929 ± 0.375	0.991 ± 0.533
IL-2	4.394 ± 5.174	1.556 ± 0.732	3.017 ± 2.459	2.426 ± 1.329	2.466 ± 1.283	2.256 ± 0.542	1.951 ± 0.608	1.736 ± 0.790	3.511 ± 2.011
IL-4	1.838 ± 1.905	0.423 ± 0.069	1.038 ± 0.615	0.709 ± 0.443	0.680 ± 0.292	0.530 ± 0.099	0.523 ± 0.155	0.506 ± 0.179*	1.077 ± 0.317*
IL-5	4.601 ± 1.879	2.309 ± 0.385*	3.404 ± 0.665*	6.395 ± 1.826	38.674 ± 30.874*	57.238 ± 64.938	2.869 ± 0.598	72.351 ± 112.187	36.086 ± 55.504
IL-6	12.631 ± 9.597	6.000 ± 1.227	17.583 ± 2.641*	13.017 ± 3.871	19.536 ± 15.370	16.414 ± 7.413	7.920 ± 2.733	32.420 ± 44.725	60.384 ± 51.114
IL-9	0.578 ± 0.247	0.225 ± 0.062*	0.468 ± 0.295	0.443 ± 0.193	4.880 ± 7.537	2.741 ± 2.179*	0.264 ± 0.069	5.908 ± 8.053	4.314 ± 6.227
MCP1	64.399 ± 11.538	60.809 ± 16.146	60.158 ± 17.366	105.631 ± 40.350	795.294 ± 583.575*	503.138 ± 338.957*	51.460 ± 11.365	716.211 ± 536.874*	1003.764 ± 769.977*
MIP1α	1.516 ± 0.761	1.058 ± 0.128	1.409 ± 0.418	1.050 ± 0.140	1.843 ± 1.348	1.215 ± 0.299	1.260 ± 0.216	2.645 ± 2.647	7.410 ± 5.870 *
MIP1β	13.271 ± 1.121	11.505 ± 2.406	12.013 ± 3.559	11.958 ± 2.031	27.606 ± 19.550	17.200 ± 5.564	14.981 ± 0.908	55.024 ± 53.613	124.403 ± 94.232 *
TNF-α	3.488 ± 1.461	2.613 ± 0.481	2.928 ± 0.500	5.446 ± 3.625	24.823 ± 27.422	9.355 ± 4.844	3.478 ± 0.662	40.976 ± 47.749	80.643 ± 88.224

Each data point represents the mean and standard deviations. Statistically significant values are denoted by * ($p<0.05$).

(CXCL1) Chemokine C-X-C motif ligand 1, (GM-CSF) Granulocyte-macrophage colony-stimulating factor, (IFN-γ) Interferon gamma, (IL-1β) Interleukin 1 beta, (IL-10) Interleukin 10, (IL-12p70) Interleukin 12, (IL-13) Interleukin 13, (IL-17A) Interleukin 17A, (IL-2) Interleukin 2, (IL-4) Interleukin 4, (IL-5) Interleukin 5, (IL-6) Interleukin 6, (IL-9) Interleukin 9, (MCP1) Monocyte chemoattractant protein 1, (MIP1α) Macrophage Inflammatory Protein-1 alpha, (MIP1β) Macrophage Inflammatory Protein-1 beta, (TNF-α) Tumor necrosis factor-alpha

Chapter 2: Research Note Exploring the competence of various poultry species for Cache Valley virus infection

Authors: Krisangel López[*], John A. Muller[*], Manette Tanelus[*], Dawn I. Auguste[*], William B. Stone[*], Sally Paulson[*], Amy Rizzo[†], Chad E. Mire[§], Albert J. Auguste[*,‡,1]

Author Affiliations: [*]Department of Entomology, Fralin Life Science Institute, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061; [†]Animal Resources and Care Division, Office of Research, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061; [§]United States Department of Agriculture, Agricultural Research Services, National Bio and Agro-defense Facility, Foreign Arthropod-Borne Animal Diseases Research Unit, Manhattan, Kansas, 66502; [‡]Center for Emerging, Zoonotic, and Arthropod-borne Pathogens, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061.

[1]**Address for Correspondence**: *Albert J. Auguste, Department of Entomology, College of Agriculture and Life Sciences, Fralin Life Science Institute, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061. Phone Number: 540-231-6158 Email: jauguste@vt.edu

I (Krisangel López) am first author of the following manuscript and give permission to use this work, provided that proper citations are accredited to the authors.

Chapter 2: Exploring the competence of various poultry species for Cache Valley virus infection

Authors: Krisangel López[*], John A. Muller[*], Manette Tanelus[*], Dawn I. Auguste[*], William B. Stone[*], Sally Paulson[*], Amy Rizzo[†], Chad E. Mire[§], Albert J. Auguste[*,‡,1]

Author Affiliations: [*]Department of Entomology, Fralin Life Science Institute, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061; [†]Animal Resources and Care Division, Office of Research, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061; [§]United States Department of Agriculture, Agricultural Research Services, National Bio and Agro-defense Facility, Foreign Arthropod-Borne Animal Diseases Research Unit, Manhattan, Kansas, 66502; [‡]Center for Emerging, Zoonotic, and Arthropod-borne Pathogens, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061.

[1]**Address for Correspondence**: *Albert J. Auguste, Department of Entomology, College of Agriculture and Life Sciences, Fralin Life Science Institute, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061. Phone Number: 540-231-6158 Email: jauguste@vt.edu

Key words: Cache Valley Virus, Bunyaviruses, Growth Curves, Animal Models

Scientific Section: Animal Well-Being and Behavior; Immunology, Health and Disease; Molecular and Cellular Biology; Microbiology and Food Safety.

Journal: Poultry Science; *in review*

Abstract

Cache Valley virus (CVV) belongs to the genus *Orthobunyavirus*, and is known to cause severe disease in ruminants, including spontaneous abortions and congenital defects. Previous evidence suggests there is the potential of CVV to infect poultry species due to its wide geographic range, reports of seropositivity in birds for Cholul or Maguari virus (closely related viruses), and isolations of CVV from highly ornithophilic mosquito vectors. To determine CVV's potential as a disease-causing agent in poultry species, we used two strains from the two recognized genetic lineages of CVV for both our in-vivo and in-vitro studies. We assessed CVV's growth kinetics in three avian cells lines, including domestic chicken (*Gallus gallus*; DF-1), Japanese quail (*Coturnix coturnix japonica:* QNR/K2*),* and Pekin Duck cells (*Anas platyrhynchos domesticus:* PDE). For the in-vivo studies, we challenged three-day old SPF-chickens (*Gallus gallus*), three-day old ducklings (*Anas platyrhynchos domesticus*), and 14-day old quail (*Coturnix coturnix)* with both CVV strains. We found that CVV grew rapidly and to high titers in all three avian cell lines yet failed to induce a symptomatic infection during in-vivo studies. Our data suggests that domestic poultry species are likely not significant contributors to the maintenance of CVV.

Introduction

Cache Valley virus (CVV) belongs to the genus *Orthobunyavirus* in the family *Peribunyaviridae*. Initially discovered in Cache Valley, Utah, in 1956 (Holden & Hess, 1959), CVV is known to cause severe disease in ruminants, including spontaneous abortions and congenital defects (i.e., arthrogryposis) (Hughes et al., 2023; Waddell et al., 2019). Although rare, CVV has been shown to also cause severe disease in humans, including encephalitis, multiorgan failure, macrocephaly in infants, and death (Hughes et al., 2023). CVV is widespread across North America and multiple outbreaks have been reported across the United States, Canada, and Mexico (Hughes et al., 2023). CVV is also unique in that it has been detected in over 44 mosquito species, and the virus has been isolated from several ornithophilic mosquitoes including *Culex tarsalis*, *Culex pipiens, Culex quinquefasciatus,* and *Culex restuans* (Waddell et al., 2019).

CVV has been serologically detected or isolated from a wide variety of wildlife and livestock (reviewed in Waddell et al., 2019). Although the primary reservoir host for CVV remains unknown, white-tailed deer *(Odocoileus virginianus*) are suspected as the primary sylvatic host (Hughes et al., 2023). Holden and Hess (1959) challenged 0.5-day-old domestic chicks (*Gallus gallus*) with CVV which resulted in no observed viremia or neutralizing antibodies, suggesting that birds might not be an amplifying host for CVV. However, Belle, Grant, & Griffiths (1966) showed that various species of birds in Jamaica including wild birds and domestic chickens were serologically positive for CVV. Since this study was conducted, the Caribbean and South American strain of CVV has been reclassified as Maguari virus (MAGV) (Hughes et al., 2023). A follow-up survey conducted in the Yucatan peninsula to identify *Orthobunyavirus* presence in poultry and domestic animals (Blitvich et al., 2012), found no antibodies to CVV in either chicken or turkeys. However, they did find antibodies for Cholul virus (CHLV) (a natural recombinant of

CVV and Potosi virus) in turkeys (Blitvich et al., 2012). This again prompts the question of the potential agricultural impact that CVV may have in North American poultry, as no true surveys or experiments have been conducted in poultry since the reclassification of MAGV and CHLV.

Altogether, given the seropositivity in birds for MAGV and CHLV which are viruses within the same Bunyamwera serogroup, the isolation of CVV from highly ornithophilic mosquito vectors, the strong association CVV has with livestock, we sought to experimentally assess CVV infection in agriculturally important poultry species by conducting both *in vitro* and *in vivo* studies to evaluate viral growth in avian cell cultures and the progression of disease in various poultry species.

Materials and Methods

Viruses and Cell Culture

Strains from two genetic lineages of CVV were used in the study: CVV W08491 from Lineage 1 (provided by Dr. Philip Armstrong at the Connecticut Agricultural Experiment Station) and CVV 4B from Lineage 2. Strains were selected based on their genetic diversity, passage history, and enzootic sources. CVV W08491 was isolated from a pool of *Culex tarsalis* (an ornithophilic species) mosquitoes from North Dakota in 2005 and propagated to a viral titer of 1.09×10^7 plaque forming units (PFU)/mL in Vero-76 cells (African Green Monkey Kidney clone) (ATCC; Manassas, VA, USA). CVV 4B was isolated from an *Aedes japonicus* mosquito pool from Blacksburg, Virginia in 2015, and grown to a viral titer of 7.7×10^7 PFU/mL in Vero-76 cells. The cells were grown to 80% confluent monolayers and an aliquot of each virus was added and observed for cytopathic effect (CPE). Upon observation of 50% CPE, the virus was harvested, typically about 2 days post-infection (DPI). The supernatant was collected and clarified by

centrifugation for 10 minutes at 3345 × g at a temperature of 4°C, and then stored at -80°C. Viral titers were estimated by plaque assays on Vero-76 cells as previously described (Lopez et al., 2021).

CVV growth curves in chicken, duck, and quail cell culture

We used three commercially available avian cells lines; domestic chicken (*Gallus gallus*; DF-1), Japanese quail (*Coturnix coturnix japonica:* QNR/K2), and Pekin Duck cells (*Anas platyrhynchos domesticus:* PDE) obtained from Dr. Nisha Duggal at Virginia Tech. The cell lines were grown in T-25 flasks in triplicate to ~80% confluency and kept at 39°C. Cells were infected with a multiplicity of infection (MOI) of 0.1 PFU/cell. A phosphate buffered saline (PBS) wash (x3) was conducted prior to the initial time point collection to remove any residual virus and supernatant samples were collected for each cell line at 0-, 3-, 6-, 12-, 24-, 36-, 48-, 72-, 96-, and 120-hours post-infection (HPI). All samples were run in triplicate and virus titers quantified using plaque assays as shown in Figure 1A.

CVV pathogenesis in chickens, ducks, and quail

Three-day old SPF-chickens (*Gallus gallus*) (n=18) purchased from Charles River laboratories (Wilmington, MA, USA) were subcutaneously (s.c.) inoculated with 10^4 PFU of either CVV strain (i.e., 4B [n=18] and W08491 [n=18]) or PBS (n=18). Three-day old ducklings (*Anas platyrhynchos domesticus*) purchased from Murray McMurray Hatchery (Webster City, IA) were also s.c. inoculated with 10^4 PFU of either CVV strain (i.e., 4B [n=18] and W08491 [n=18]) or PBS (n=16). Quail eggs were purchased from AJ Farms LLC (Strasburg, VA, USA). Eggs were incubated for 18 days based on manufacturer recommendations and monitored for hatching. Upon hatch, quail

were transferred to isolators and at two weeks old were s.c. inoculated with 10^4 PFU of either CVV W08491 (n=11), CVV 4B (n=11), or PBS (n=9). All infected birds were monitored daily for symptoms of disease for 14 days, brachially bled on days 1–4, and a subset (n=3) were sacrificed 3, 6, and 9 DPI for tissue collection as described in Figure 2A. Tissues were stored in culture media (i.e., DMEM containing 2% FBS, 100 units of Penicillin, and 0.1 mg Streptomycin) for virus quantification by plaque assay. Sera collected at the study's termination day (i.e., 14 DPI) were used for 50% plaque reduction neutralization tests ($PRNT_{50}$) to measure neutralizing antibody responses against respective virus strains (4B and W08491). For the $PRNT_{50}$ assay, sera samples were heat inactivated for one hour at 56°C to inactivate proteins that could interact with the identification of the antibodies of interest. Samples were then diluted using 2% DMEM (components described above) and two-fold serially diluted to generate sera dilutions ranging from 1:20 to 1:640.

A virus only control plate consisting of 100 PFU of virus stock was diluted with equal parts virus stock and 2% DMEM (components described above) and used for each batch of $PRNT_{50}$ assays. Virus-serum mixtures were incubated for one hour at 37°C before being plated in Vero-76 cells. Once plated, a second one-hour incubation followed at 37°C, before a 0.4% agarose overlay (components described above) was added. Plates were fixed 2 DPI using 10% formaldehyde and visualized using 0.2% crystal violet.

Statistical Analysis

The data is depicted as mean ± Standard Deviation (SD). To determine differences between groups, samples were analyzed using Logrank Mantel-Cox tests to assess significance among survival, and

t-tests to assess significance among weight data. Statistical significance is depicted as a p-value of <0.05. All statistical tests were performed using GraphPad Prism Version 10.2.3

Ethics Statement

This study was performed under IACUC Protocol 20-071, which was approved by Virginia Tech's IACUC on 4/9/2020.

Results and Discussion

In this study, we report the first comprehensive laboratory studies of CVV infection in poultry since the initial study conducted in chickens (Holden & Hess, 1959). In addition to working with chickens, we also explored the potential effects of CVV in two additional poultry species, including domestic ducks and Japanese quail. CVV grew rapidly and to high titers in these avian cell lines yet failed to induce a symptomatic infection in any of the poultry species used here. This data suggests that there is a component of the avian immune system that makes these poultry species resistant to CVV induced disease. A previous study demonstrated that mice lacking the alpha and beta type-1 interferon receptor developed viremia and significant disease when infected with CVV, however, the wildtype/immune-competent mouse model did not (Lopez et al., 2021). This suggests that the avian innate immune response could be playing a significant role in the viruses' ability to cause infection and disease and could be preventing the virus from spilling over into poultry. Further, studies to assess how the avian immune system might evade CVV infection are warranted to better assess potential transmission and spillover potential.

CVV grows efficiently in avian cell lines

To determine if CVV can replicate in avian cell cultures, we infected three commercially available avian cell lines (DF-1, QNR/K2, and PDE) with two contemporary strains of CVV (4B and W08491) representing both genetic lineages (Figure 1A). Our data shows that, regardless of the viral strain used, CVV grows efficiently in all three cell lines. A significant increase in viral titer is observed in all three cell lines by 12 HPI (Figure 1B-D). In DF-1 cells, CVV replicates exponentially until 48 HPI with a peak viral titer of 8.06 $\log_{10}$ (± 0.14 SD) for CVV 4B and 5.06 $\log_{10}$ (± 0.03 SD) PFU/mL for CVV W08491. CVV plateaus and maintains a titer of 6.38 $\log_{10}$ (± 0.25 SD) for 4B and 5.9 $\log_{10}$ (± 0.07 SD) for CVV W08491 at 120 HPI (Figure 1B). QNR/K2 shows peak viral growth at 72 HPI, with a viral titer of around 6.43 $\log_{10}$ (±0.34 SD) for CVV 4B, and 6.4 $\log_{10}$ (±0.09 SD) for CVV W08491, which remains consistent until the end of the study (Figure 1C). In PDE cells, CVV achieved peak titers earlier than the other two cell lines by 36 HPI, however, the overall titers are lower at 4.91 $\log_{10}$ (± 0.08 SD) for CVV 4B and 5.55 $\log_{10}$ (±0.05 SD) for CVV W08491 which remains consistent until the end of the study (Figure 1D). Although all of the cell lines showed small differences in growth, DF-1 was the only cell type that shows significant growth differences between viral strains, with CVV 4B (Lineage 2) having a four-log difference when compared to the ancestral lineage 1, CVV W08491 (Figure 1B). This discrepancy could be explained by the immunological differences and cell types within these cell lines (DF-1 and PDE are fibroblasts and QNR/K2 are neuroretina). Previous studies have presented significantly different responses to viral infections even between poultry species within the same taxonomic order (e.g., Pekin duck [*Anas platyrhynchos*] and Muskovy ducks [*Cairina moschata*]) (Schultz & Magor, 2022). Further studies into the immunological differences and cell types between these cell lines are warranted to explore the differences in viral replication patterns observed in this study.

CVV does not cause morbidity or mortality in poultry species

To explore the pathogenesis of CVV in poultry species and potential differences in strain-specific pathogenesis, 3-day old chickens, 3-day old ducks, and two-week old quail were subcutaneously inoculated with either CVV W08491 or 4B, or PBS diluent (Figure 2A). Birds were monitored for 14 DPI and overall, no significant weight loss (Figure 2B-2D) or mortality (Figure 2E-2G) was observed when compared to healthy controls. To measure viremia in infected birds, blood was collected 1–4 DPI and virus titers quantified by plaque assay. No viremia was detected among any of the six infected CVV groups (data not shown; limit of detection is 100 PFU). To assess serological status in birds at the study's end, blood was collected 14 DPI and sera harvested for $PRNT_{50}$ testing. CVV failed to induce any detectable neutralizing antibody response in any of the infected birds (limit of detection being 1:20 $PRNT_{50}$ dilution). Altogether, our results show that CVV grows well in avian cell culture systems but failed to illicit symptomatic infections or neutralizing antibody responses in any of the poultry species studied. These experimental infection data suggests that CVV may be an unlikely pathogen for poultry species. Future studies are needed and should also include a Caribbean and Central/ South American strain of CVV, such as Maguari virus (Groseth et al., 2017), or Cholul virus which is a recombinant strain of CVV and Potosi virus, that has been previously isolated from turkeys (Blitvich et al., 2012). These studies could help determine if there are virus-specific restrictions preventing CVV from causing a systemic and symptomatic infection in birds. Additionally, a previous study conducted in mice, showed that CVV did not produce an infection in immune competent mice, however, adding mosquito saliva to the inoculum elicited a symptomatic infection (Edwards, Higgs, & Beaty, 1998). Future studies are necessary with mosquito saliva in the inoculum or using mosquitoes for direct transmission may help induce a symptomatic infection in these bird species.

Although CVV did not induce disease or neutralizing antibody responses in the poultry species tested here, there remains the possibility that CVV may be able to replicate and cause disease in non-poultry avian species. Belle et al. (1966) showed that various wild passerine species tested serologically positive for CVV. Experimental infections of various passerine species (such as house sparrows, zebra finches or canaries) are needed to determine if passerines can play a role in the maintenance and dispersal of CVV both locally and potentially internationally during long-distance migration (Belle et al., 1966; Reed, Meece, Henkel, & Shukla, 2003).

In summary, our data suggests it is unlikely that domestic poultry species are significant contributors to the maintenance of CVV within agricultural systems or will suffer significant disease as a result of CVV infection. However, further transmission studies, the use of other closely related viruses (i.e., Cholul or Maguari virus), and exploring competence in passerines are necessary to determine if CVV has the potential to expand into other avian species.

ACKNOWLEDGEMENTS

This work was partially supported by an integrated seed grant from the Center for Advanced Innovation in Agriculture, College of Agriculture and Life Sciences at Virginia Tech. This work was also partially supported by a grant from National Institute of Allergy and Infectious Diseases of the National Institutes of Health under award number U19AI181968 to AJA. KL was supported by a Gilliam Fellowship for Advanced Study from the Howard Hughes Medical Institute. MT was supported by a George Washington Carver Fellowship from the College of Agriculture and Life Sciences at Virginia Tech. This work was also partially supported by a contract from the United States Department of Agriculture (USDA) Agricultural Research Service under agreement #58-3022-4-044 to AJA. AJA was supported by the USDA National Institute of Food and Agriculture,

Hatch VA-160103, project 1020026. We would like to thank Dr. Nisha Duggal for sharing the avian cell lines used in this study and Dr. Phillip Armstrong for providing original viral stock of CVV W08491. Figure schematics were created using Biorender.com.

DISCLOSURES

The authors of this publication declare no conflict of interest.

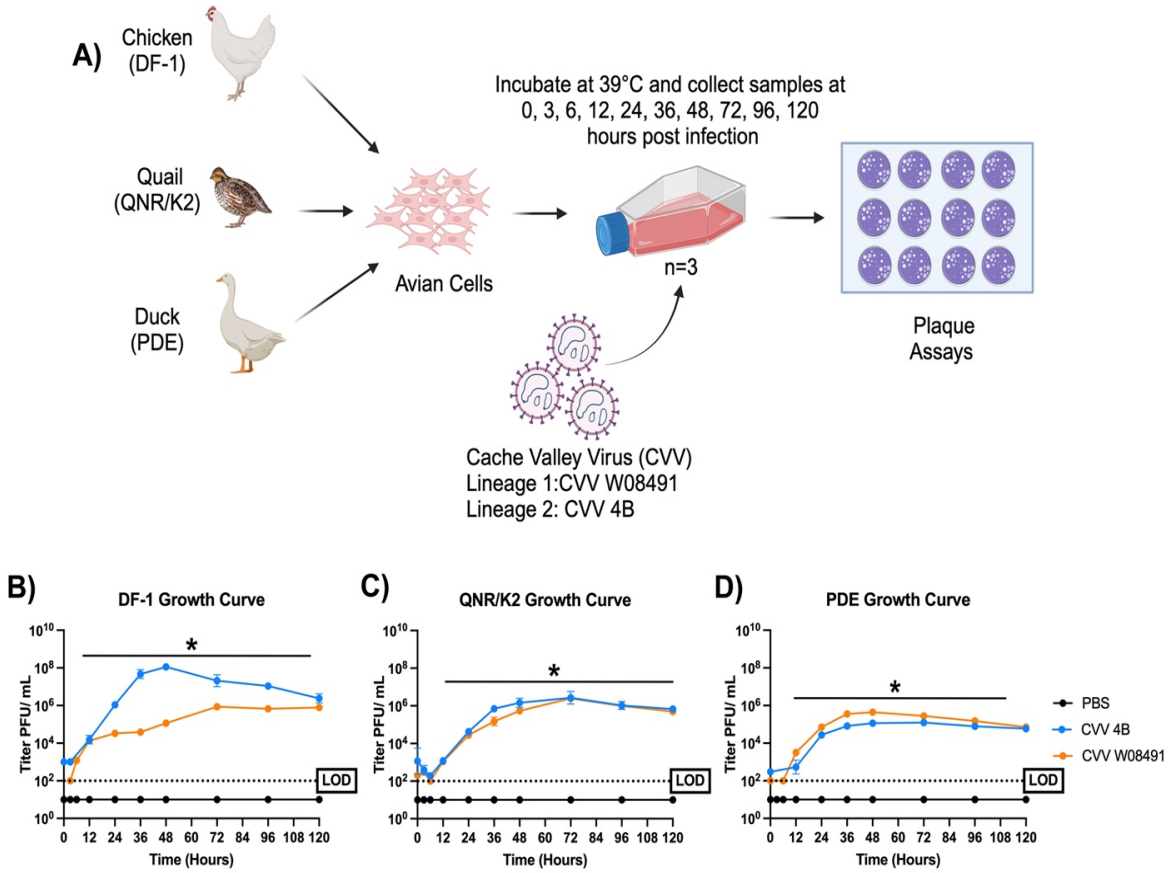


Figure 1. Cache Valley virus (CVV) replicates efficiently in avian cell culture systems. (A) Schematic of the study design used to assess CVV replication in avian cell lines. The three cell lines used are depicted by the corresponding species they were harvested from, and the type of cell used. Flasks were infected with either CVV W08491 (Lineage 1; in orange) or CVV 4B (Lineage 2; in blue), or Negative Control: PBS (Phosphate Buffered Saline; in black) and incubated for 120 hours at 39°C. Samples were run in triplicate and quantified using plaque assay on Vero-76 cells. Viral growth kinetics are shown in (B) DF-1 (*Gallus gallus*), (C) QNR/K2 (*Coturnix coturnix japonica*), and (D) PDE (*Anas platyrhynchos domesticus)* cells. Each data point represents the mean values, and the error bars represent the standard deviation. Limit of detection (LOD) is depicted by the dotted line. Statistical significance among the groups was analyzed using t-tests (B-D). Statistically significant values are denoted by *(p<0.05).

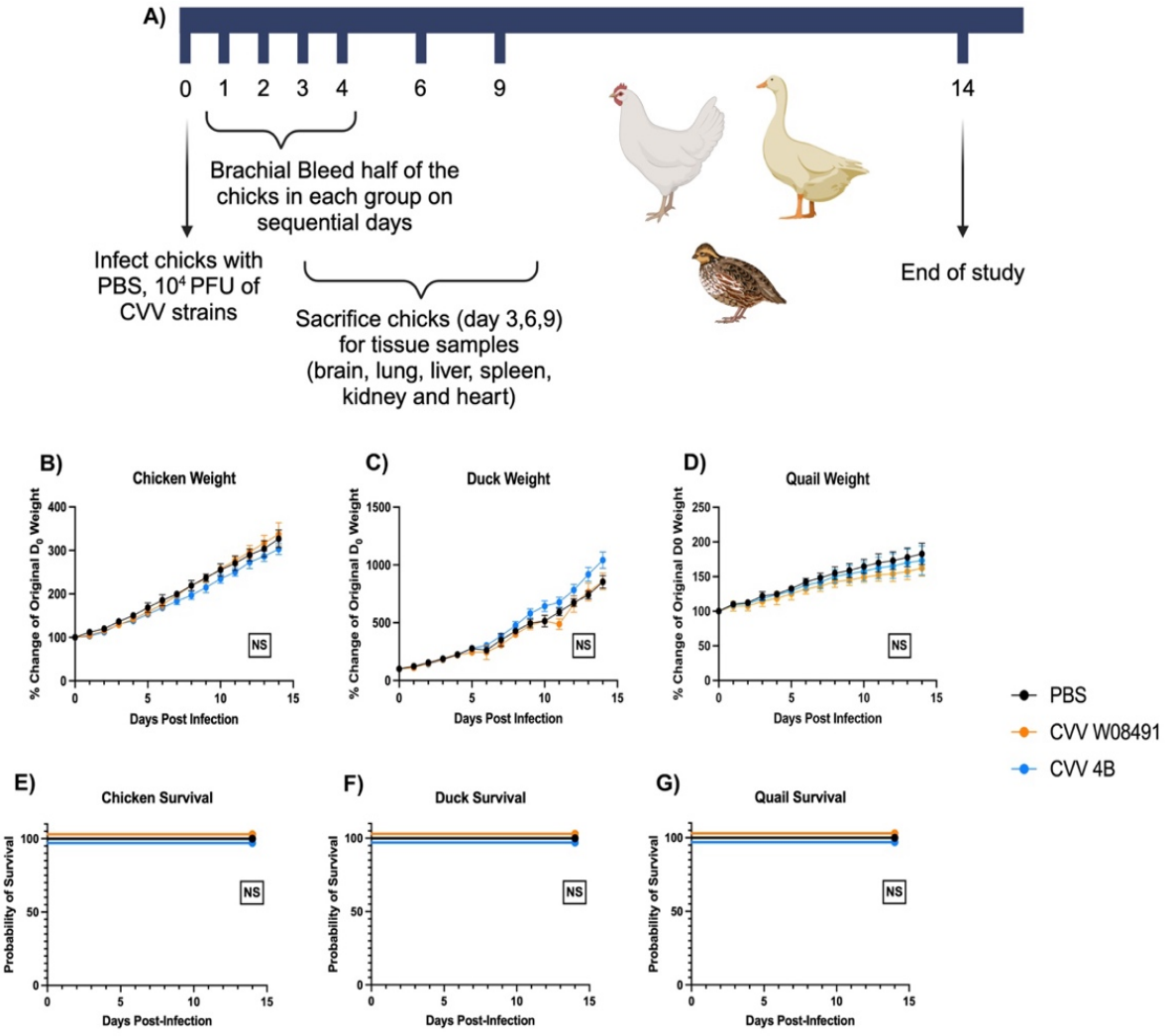


Figure 2. Cache Valley virus (CVV) infection does not induce weight loss or mortality in poultry. (A) Schematic of the *in vivo* study procedure. Three-day old chickens (CVV 4B, CVV W08491, PBS; n=18), 3-day old ducks (CVV 4B n=18, CVVW08491 n=17, PBS=16) , and two-week old quail (CVV 4B n=11, CVVW08491 n=11, PBS=9) were subcutaneously inoculated with 10^4 plaque forming units (PFU) of either CVV W08491 (Lineage 1; in orange), CVV 4B (Lineage 2; in blue), or Negative Control: PBS (Phosphate Buffered Saline; in black), and monitored for 14 days post infection for changes in weight (B-D) and survival (E-G). Each data point represents the mean values, and the error bars represent the standard deviation. Statistical significance among the groups was analyzed using t-tests (B-D) and log-rank (Mantel-Cox) in (E-G). Statistically significant values are denoted by *($p<0.05$). Non-significant values are denoted as NS.

References

Belle, E. A., Grant, L. S., & Griffiths, B. B. (1966). The isolation of Cache Valley virus from mosquitoes in Jamaica. *West Indian Med J, 15*(4), 217-220.

Blitvich, B. J., Saiyasombat, R., Travassos da Rosa, A., Tesh, R. B., Calisher, C. H., Garcia-Rejon, J. E., . . . Lorono-Pino, M. A. (2012). *Orthobunyaviruses*, a common cause of infection of livestock in the Yucatan peninsula of Mexico. *Am J Trop Med Hyg, 87*(6), 1132-1139.

Edwards, J. F., Higgs, S., & Beaty, B. J. (1998). Mosquito Feeding-Induced Enhancement of Cache Valley Virus (*Bunyaviridae*) Infection in Mice. *Journal of Medical Entomology, 35*(3), 261-265.

Groseth, A., Vine, V., Weisend, C., Guevara, C., Watts, D., Russell, B., . . . Ebihara, H. (2017). Maguari virus associated with human disease. *Emerging Infectious Diseases, 23*(8), 1325.

Holden, P., & Hess, A. D. (1959). Cache Valley virus, a previously undescribed mosquito-borne agent. *Science, 130*(3383), 1187-1188.

Hughes, H. R., Kenney, J. L., & Calvert, A. E. (2023). Cache Valley virus: an emerging arbovirus of public and veterinary health importance. *J Med Entomol, 60*(6), 1230-1241.

López K., Wilson S. N., Coutermash-Ott S., Tanelus M., Stone W. B., Porier D. L., Auguste D. I., Muller J. A., Allicock O. M., Paulson S. L., Erasmus J. H., & Auguste A. J. (2021). Novel murine models for studying Cache Valley virus pathogenesis and *in utero* transmission. *Emerg Microbes Infect*, 10(1), 1649-1659.

Reed, K. D., Meece, J. K., Henkel, J. S., & Shukla, S. K. (2003). Birds, migration and emerging zoonoses: West Nile virus, Lyme disease, influenza A and enteropathogens. *Clin Med Res, 1*(1).

Schultz, U., & Magor, K. E. (2022). Comparative immunology of agricultural birds. In *Avian immunology* (pp. 489-518).

Waddell, L., Pachal, N., Mascarenhas, M., Greig, J., Harding, S., Young, I., & Wilhelm, B. (2019). Cache Valley virus: A scoping review of the global evidence. *Zoonoses Public Health, 66*(7), 739-758.

Chapter 3: Mosquito surveillance across Virginia demonstrates range expansion of invasive mosquito species and the isolation of several arboviruses.

Authors: Krisangel López[1], John A. Muller[1], Dawn I. Auguste[1], Manette Tanelus[1], Sally L. Paulson[1], Luis E. Escobar[2,9], Joanna M. Reinhold[3], Chloe Lahondere[3,9], Orchid M. Allicock[4], Lin Kang[5,6,7], Christine V.F. Carrington[8], Albert J. Auguste[1,9]

Author Affiliations: [1] Department of Entomology, Fralin Life Science Institute, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061; [2] Department of Fish and Wildlife Conservation, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061; [3]Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061; [4]Department of Epidemiology of Microbial Diseases, Yale School of Public Health, New Haven, CT, USA; [5]Deparment of Biomedical Sciences Research, Edward Via College of Osteopathic Medicine, Monroe, LA, USA; [6]Department of Biomedical Sciences and Pathobiology, Virginia-Maryland College of Veterinary Medicine, Blacksburg, VA, USA; [7]College of Pharmacy, University of Louisiana Monroe, Monroe, LA, USA; [8]Department of Preclinical Sciences, Faculty of Medical Sciences, The University of the West Indies; [9]Center for Emerging Zoonotic, and Arthropod-Borne Pathogens, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061

I (Krisangel López) am first author of the following manuscript and give permission to use this work, provided that proper citations are accredited to the authors.

Chapter 3: Mosquito surveillance across Virginia demonstrates range expansion of invasive mosquito species and the isolation of several arboviruses.

Authors: Krisangel López[1], John A. Muller[1], Dawn I. Auguste[1], Manette Tanelus[1], Sally L. Paulson[1], Luis E. Escobar[2,9], Joanna M. Reinhold[3], Chloé Lahondère[3,9], Orchid M. Allicock[4], Lin Kang[5,6,7], Christine V.F. Carrington[8], Albert J. Auguste[1,9]

Author Affiliations: [1] Department of Entomology, Fralin Life Science Institute, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061; [2] Department of Fish and Wildlife Conservation, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061; [3]Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061; [4]Department of Epidemiology of Microbial Diseases, Yale School of Public Health, New Haven, CT, USA; [5]Deparment of Biomedical Sciences Research, Edward Via College of Osteopathic Medicine, Monroe, LA, USA; [6]Department of Biomedical Sciences and Pathobiology, Virginia-Maryland College of Veterinary Medicine, Blacksburg, VA, USA; [7]College of Pharmacy, University of Louisiana Monroe, Monroe, LA, USA; [8]Department of Preclinical Sciences, Faculty of Medical Sciences, The University of the West Indies; [9]Center for Emerging Zoonotic, and Arthropod-Borne Pathogens, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061

Journal Targeted: *Emerging Infectious Diseases*

Abstract

Arthropod-borne pathogens are among the leading causes of human morbidity and mortality worldwide. As we start to experience the effects of climate change and the range expansion of important mosquito vectors, establishing a baseline for vector and pathogen diversity is fundamental to develop effective disease management strategies. To address this challenge, I assessed the vector and pathogen diversity across distinct ecosystems. The Commonwealth of Virginia has a diverse ecological landscape, spanning from coastal swamps to mountainous ranges. Three ecosystems across Virginia; including Coastal Plains (swamp), Piedmont (savannah), and Blue Ridge Mountains (primary forest) were surveyed. The mosquito surveys were conducted for one week every month between May and October 2022. Mosquitoes were collected using CO_2-baited CDC light traps and maple leaf infusion-baited gravid traps, and were sorted into pools by location, trap type, date, and species. A total of 7,247 mosquitoes were collected and sorted into 876 pools. Pools were screened for viruses via cytopathic effect assays in three cell lines (Vero-76, BHK-21, and C7/10) at three different incubating temperatures (37°C, 30°C, and 28°C). Diagnostic PCR with Pan-Flavi-, Pan-Bunya-, and Pan-Alpha- virus primers were used to identify virus families, and amplicons were sequenced to identify pathogens. I isolated one vertebrate pathogenic virus, Jamestown Canyon virus, multiple non-pathogenic vertebrate virus's (Muir Springs virus, Flanders hapavirus) and 58 insect-specific viruses including multiple *Culex* flaviviruses, Nam Dinh virus, Houston virus, and Phasi Charoen-like phasivirus. My studies report on the phylogenetic characteristics and host-pathogen associations observed during these vector surveys. Ultimately, this study will contribute to our understanding of the extensive viral diversity across Virginia and assist public health officials in developing risk assessments for Virginia and neighboring states.

Introduction

Vector-borne diseases (VBDs) account for approximately 17% of the infectious disease burden globally (Romero-Alvarez, Escobar, Auguste, Del Valle, & Manore, 2023; Wilson et al., 2020). It is suspected that more than 80% of the world's population is suspected to live in areas at risk of VBDs (Perrin, Glaizot, & Christe, 2022). Among disease vectors, mosquitos are considered the deadliest in the world and are responsible for millions of cases annually (Madewell, 2020). Mosquitoes transmit a variety of pathogens, including protozoans (i.e., malaria), nematodes (i.e., lymphatic filariasis), and viruses (i.e., Zika virus, yellow fever virus [YFV]) (Foster & Walker, 2019). Mosquitoes boast an expansive distribution and in the last 50 years their habitat suitability continues to grow. Climate change is known to increase the suitable conditions to induced mosquitoes range expansion globally (Rochlin, Ninivaggi, Hutchinson, & Farajollahi, 2013). Mosquitoes increased range expansion into novel regions increases the likelihood of disease emergence in naïve human populations (Lwande et al., 2020; Musso, Rodriguez-Morales, Levi, Cao-Lormeau, & Gubler, 2018).

In the last century, we have seen a global surge in arthropod-borne viruses (arboviruses) (Musso et al., 2018). For example, in the last 30 years, dengue case reports have increased by nearly 450% worldwide (Wilson et al., 2020). We have also seen a significant rise in lesser known emerging and remerging arboviruses, like St. Louis encephalitis (SLEV), La Crosse virus (LACV), Cache Valley virus (CVV), and Jamestown Canyon virus (JCV) (Madewell, 2020). Arbovirus infections can leave long-lasting sequelae in patients who contract neuroinvasive disease, and in severe cases, cause death (Pastula, Smith, Beckham, & Tyler, 2016). Although vaccine efforts have been made for a wide variety of arboviruses, these efforts have been unsuccessful, with few exceptions (e.g., Japanese encephalitis, yellow fever virus) (Metz & Pijlman, 2011; Wilson et al.,

2020). To combat the rise in arboviruses there has been an increase in insecticide spraying in many areas of the world. Consequently, this has resulted in mosquitoes developing resistance to insecticides, posing challenges in controlling vector populations and the spread of mosquito-borne diseases (Liu, 2015; Metz & Pijlman, 2011). Because of the compounding effects of climate change-driven increased habitat suitability for vectors, increased insecticide resistance, and an expansion in arbovirus prevalence over recent years, there is a need for monitoring strategies such as viral and mosquito surveillance (Wilson et al., 2020).

Mosquito surveillance is a key part of developing proper disease-mitigating strategies in conjunction with techniques such as vector control and public health management (Schwab, Stone, Fonseca, & Fefferman, 2018). Surveillance surveys are critical information tools that aid in understanding species presence and absence, habitat and species composition, and geographic and seasonal distribution. Monitoring of these factors is necessary to develop proper risk assessments for disease management. Studies have shown that lapses in surveillance have led to a huge resurgence of mosquito populations (Wilson et al., 2020). A great example of this is the eradication programs in South America for *Aedes aegypti*. Historically, using surveillance and vector eradication programs in the 1950s and 1960s, 16 South American countries were able to eradicate *Aedes aegypti* populations (Soper, 1963). Nevertheless, leniency regarding surveillance and vector control allowed this mosquito to recolonize rapidly leading to large epidemics of multiple arboviruses (Soper, 1963; Wilson et al., 2020). Approximately 91% of the malaria cases since the resurgence of *Aedes aegypti* is due to lax surveillance and vector control efforts (Cohen et al., 2012).

The Commonwealth of Virginia has unique landscape features and high biodiversity, making it an ideal region to conduct mosquito surveys across ecosystems. Virginia has five distinct

eco-regions, including the Coastal Plains, Piedmont, Blue Ridge Mountains, Valley and Ridge, and the Appalachian Plateau (Virginia Museum of History & Culture, 2024). Having a diverse landscape allows for critical observations on how changes in landscape features affect mosquito and viral diversity across eco-regions. There is limited knowledge regarding the scope of arboviral and mosquito diversity across the Commonwealth of Virginia. Most mosquito and arboviral surveillance in Virginia has been biased to three major metropolitan areas in the eastern, areas of the state (i.e., Fairfax, Richmond, and Suffolk) (Loftin, Herbert, & Phaltankar, 2006; Virginia Department of Health, 2019). In addition, state surveillance is also limited in the number of arboviruses screened, usually only Eastern Equine Encephalitis virus (EEEV) and West Nile (WNV) viruses (Loftin et al., 2006; Virginia Department of Health, 2019). Although EEEV and WNV are important pathogens, they are not the only relevant arboviruses of concern in the eastern United States, and having a better understanding of arboviral diversity can aid public health officials in protecting local citizens. To further advance arboviral surveillance, we conducted a survey of both mosquitoes and viruses focused in three different eco-regions. Our study demonstrates the high diversity of both mosquito and viral species across the state of Virginia.

Methods

Study Area

Three wildlife management areas (WMA) were used in the study, to represent coastal plains, Piedmont, and Valley and Ridge ecoregions. Site 1, Cavalier WMA is a conservation area located in Chesapeake County, southeastern corner of Virginia. Cavalier WMA covers approximately 4,550 acres and consists of two tracks, the 750-acre Dismal Swamp tract, and the 3,800-acre Cavalier tract which abuts the North Carolina State line (Figure 1). Cavalier WMA has a wide

diversity of habitats including coastal, forested, swamp, and seasonal wetlands. There is an abundance of wildlife present at the site including black bears (*Ursus americanus*), Neotropical migratory songbirds, Canebrake rattlesnakes (*Crotalus horridus*), white-tailed deer (*Odocoileus virginianus*) and eastern turkeys (*Meleagris gallopavo*) (Virginia Department of Wildlife Resources, 2024b). Site 2, Featherfin WMA, spans nearly 2,800 acres and spreads across three different counties (i.e., Prince Edward, Appomattox, and Buckingham County) (Figure 1). Featherfin WMA features diverse habitats including forested ridges, small drainages, extensive wetlands along the Appomattox River, row crop agricultural areas, and planted loblolly pine stands (Virginia Department of Wildlife Resources, 2024c). Site 3, Big Survey WMA, is approximately 7,500 acres located south of Wytheville, VA in Wythe County (Figure 1). The area features four mountain ridges and divides Reed Creek and Cripple Creek watersheds in the New River Valley. The landscape is very rugged dominated by oaks, hickories, and yellow pine trees with notable quartzite outcroppings, and does not support any perennial streams. Wildlife found in the site Big Survey WMA includes black bears, turkey squirrel (*Sciurus anomalus*), ruffed grouse (*Bonasa umbellus*), and white-tailed deer (Virginia Department of Wildlife Resources, 2024a).

Sample collection

Mosquito collections were conducted once per month (three days per site per trip) for five months from May 15th, 2022, until October 1st, 2022. We used four Carbon dioxide (CO_2)-baited CDC light traps (BioQuip Products, Rancho Dominguez, CA) and maple leaf infused water baited Gravid traps (BioQuip Products) at each site. In addition to the four sets of traps, we also used vacuum sampling to collect the mosquitoes. Traps were set at dusk each night and collected at dawn the following morning. Each set of traps was placed at least 100 meters from the other sets

to ensure sufficient distance to avoid sampling biases (Figure 2). Upon collection, mosquitoes were separated into containers based on location, trap type, trap number, and day of collection. These were then placed in a transportation cooler with dry ice to preserve the specimens, transported back to the lab, and placed in a -80°C freezer until further processing**.**

Sample processing

Mosquitoes in each container were sorted into pools based on species using a dissecting scope and the North American Mosquito guide (Darsie & Ward, 2005). The mosquito pools were added to 2 mL homogenizing tubes with sterile metal beads and 1,000 ml of Dulbecco's Modified Eagle Media (DMEM) containing 2% fetal bovine serum (FBS), 100 units of Penicillin, 0.1 mg Streptomycin, 1% Non-Essential Amino Acids, and 1% Amphotericin B, and kept on ice to prevent deterioration of the sample (Figure 3). Mosquito pools were homogenized at 30,000Hz/min for five minutes (cassettes were chilled at -20°C prior to use to maintain cold chain). The pools were then clarified via centrifugation at 15,000 RPM for ten minutes at 4°C. A total of 500ml of supernatant was transferred to a clean 1.5ml Eppendorf tube which was spun again for an additional 10 minutes as described above. 100ml of each sample was added to each of three different cell lines and incubated at three distinct temperatures - Vero-76 at 37°C, BHK-21 at 30°C, and C7/10 at 28°C. These cell lines were employed to increase the diversity of viruses that can be detected and/or propagated in cell culture. Once plated, the samples were rocked for one hour and a liquid media (2% DMEM as above) overlay was added before further incubation at the respective temperatures. Samples were monitored daily for signs of cytopathic effect (CPE) for a total of 14 days. Upon observation of CPE, the sample was harvested, confirmed and then stored at -80°C until further processing (Figure 4a-b).

Sample Confirmation

To rule out the effects of toxicity caused by remnant mosquito parts or fungus, samples that showed CPE during the initial screening were tested once more. A total of 100ml of the CPE positive sample was added to a T-25 flasks with fresh (~80%) confluent cells (cells used were based on the original CPE+ cell line for each individual sample). Once added, the sample was rocked for one hour and 5mL of liquid overlay was added (DMEM with 2% FBS). The samples were monitored for 14 days. Upon observation of confirmation CPE, the sample was collected and stored at -80°C until further processing could be completed.

RNA Extraction and RT-PCR

CPE positive samples were RNA extracted using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) based on manufacturer's instructions. To survey the samples as broadly as possible, we obtained primer sets for Pan-Alphavirus (Bronzoni, Baleotti, Ribeiro Nogueira, Nunes, & Moraes Figueiredo, 2005), Pan-Flavivirus (Bronzoni et al., 2005) and Pan-Bunyavirus (Dunn, Pritlove, & Elliott, 1994) (Table 1), and used a 25 mL RT-PCR reaction to amplify target relevant genes. The 25 µl reaction contained 12.5 µl of One-step ToughMix, 1 µl of the qScript XLT One-step Reverse Transcriptase (X25), (Quanta Biosciences, Gaithersburg, MD, USA) 4.5 µl of molecular-grade water, 1 µl of forward primer (Table 1) 1 µl of the reverse primers (Table 1), 1 µl of qScript XLT One-Step RT and 5 µl of sample. The amplification process was conducted using a Thermo cycler (BioRad T100 Thermal Cycler 1861096) with a 20-minute cDNA synthesis at 40°C, followed by 3-minute denaturation at 94°C and 37 cycles of 94°C for 10 seconds, 55°C for 15 seconds, 72°C for 1 minute. PCR products were visualized using a 2% agarose gel containing SYBR safe gel dye. Amplicons were cleaned up using a QIAquick gel extraction kit (Qiagen, Hilden, Germany) based

on manufacturer instructions. Samples were sent to Virginia Tech Genetics core facility for Sanger sequencing.

TRIzol Extractions and Next-Generation Sequencing

To prepare the samples for Next-Generation Sequencing (NGS), we conducted RNA extraction using TRIzol LS (Invitrogen; Thermo Fisher Scientific, Carlsbad CA, US). A total of 100ml of sample was added to 80% confluent cells in T-150 flasks (i.e., cells used corresponded to the cell line the virus was originally harvested from). These were incubated until 50% CPE was observed, then 21 ml of each cultured sample was mixed with 7 ml of 4X Polyethylene glycol (P.E.G.). The solution was mixed thoroughly and incubated overnight at 4°C. Samples were then centrifuged at 4°C at 4000 RPM for 40 minutes. The supernatant was decanted, and the pellet was resuspended in 250ml of phosphate buffered saline (PBS) solution. The resuspended P.E.G. precipitate was then mixed with 750ml of TRIzol LS solution (Invitrogen; Thermo Fisher Scientific, Carlsbad CA, US) and extracted based on manufacturer instructions, before storage at -80°C. Samples library preparation and sequencing was conducted by the Virginia Tech sequencing VT Genomic Sequencing Core, sequencing was, processed using an Illumina NovaSeq S1 with 100 single reads and 50 paired ends.

Bioinformatics

Raw data was filtered to remove sequences with low quality scores (Q<20) and exact duplicate reads using FastQC (Andrews, 2010). The ends of the reads with low quality were trimmed using Trimmomatic (Bolger, Lohse, & Usadel, 2014). Host genome reads were removed by mapping to the BHK-21 (accession GCA_017639785.1), Vero-76 (GCA_023783515.1) and *Aedes albopictus*

C7/10 (GCF_035046485.1) genomes with Bowtie2 (Langmead & Salzberg, 2012). Unmapped reads were extracted and then subjected to a basic local alignment search tool (BLASTn and BLASTx) querying the NCBI nucleotide (nt) and protein sequence database (nr) using an E-value cut-off of 1e-03 (Camacho et al., 2009). The identified viral reads were assembled by *de novo* assemblers using Megahit (Li, Liu, Luo, Sadakane, & Lam, 2015) and ABySS 2.0 (Jackman et al., 2017) to generate longer sequences. Alignment of contigs and unassembled reads to viral reference genomes was performed using Geneious Prime 2021.0.7 (https://www.geneious.com).

For the creation of the maximum likelihood phylogenetic trees, reference genomes were downloaded from NCBI. The genomes were aligned and trimmed using Geneious Prime 2023.2.1 (https://www.geneious.com). Phylogenetic trees were generated using Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-aLRT) and Ultrafast Bootstrap (UFBoot) in the program IQ-tree, and the program selected a best fit tree (Minh, Nguyen, & von Haeseler, 2013; Nguyen, Schmidt, von Haeseler, & Minh, 2014). The trees were rooted using an outgroup LACV_77 (L Segment Accession # DQ196118.1, M segment Accession # DQ196119.1, S segment Accession # DQ196120.1) and LACV_HAY539; (L Segment Accession # OP594812.1, M segment Accession # OP594811.1, S segment Accession # OP594810.1. Figtree was used to visualize and edit the trees (http://tree.bio.ed.ac.uk/software/figtree/).

Results

Mosquitoes

A total of 7,247 mosquitoes were collected, composing 34 species within 8 genera across all sites combined (Table 2). Cavalier WMA had the highest number of mosquitoes captured n=6582 whereas Big Survey WMA had the fewest with only n=201 (Table 2). Species presence varied

across sites, with 31 species found in Cavalier WMA, 14 at Featherfin WMA, and 12 at Big Survey WMA. The predominant mosquito species also varied by site. At Big Survey WMA the predominant species were *Aedes triseriatus* with 76 individuals, *Aedes. japonicus* 59, and *Culex restuans* with 38 individuals. At Cavalier WMA, the predominant species were *Culiseta inornata* with 2730 individuals, *Culex pipiens* with 1472, and *Uranotaenia sapphirina* 1030 individuals. Featherfin WMA was dominated by *Culex restuans* with 284 individuals, *Aedes vexans* 62 individuals, and *Anopheles punctipennis* with 49 individuals. We also found four invasive mosquito species including *Aedes albopictus* (found at Featherfin and Cavalier), *Aedes japonicus* (found at Big Survey, and Featherfin), *Culex coronator* (found at Cavalier), and *Culex pipiens* (found at Featherfin and Cavalier). On average, we surveyed each site three nights per month, except for Featherfin WMA, where, due to inclement weather that included storms and that damaged equipment and blocked off site access a total of only 10 nights were sampled compared to the 15 nights sampled at the other two sites.

Viruses

The 7,247 mosquitoes were divided into 876 pools based on date of collection, trap type, trap number, and species. Of the 876 mosquito pools, 77 pools were confirmed positive via the CPE assays. Of those 77 pools, 13 were from vertebrate viruses and 64 for insect-specific viruses. Of the 13 vertebrate viruses, only one was identified using RT PCR and Sanger sequencing (mosquito pool [MP] #564 [*Anopheles crucians*]). Of the remaining 12, only 2 viruses had enough detectable RNA to be submitted for Next Generation Sequencing (i.e., MP912 [*Aedes canadensis*], and MP796 [*Culiseta inornata*]). MP564 was identified both through Sanger and NGS as Jamestown Canyon Virus and/or Inkoo virus. MP796 and MP912 were both identified through NGS as

Rhabdoviruses with the most similar virus being Muir Springs virus (Accession #YP_010087313.1) and Flanders hapavirus (Accession #MH664044.1), respectively. Muir Springs was a 67% match when run through a BLAST.

Our isolate of Jamestown Canyon virus (MP564) was isolated from a pool of three *Anopheles crucians* collected at Cavalier WMA in June 2022. The L segment (Figure 5A) of our JCV isolate clustered with the JCV isolates from Massachusetts (JCV_16_MA_01; L segment Genbank Accession # MN135991.1, M segment Accession # MN135990.1, S segment Accession # MN135989.1), and Connecticut (JCV_3573-03, L Segment Accession #HM007355.1, M segment Accession # HM007354.1, S segment Accession #MH007353.1) with moderate UFbootstrap support (UFBoot of 87%). The M segment, also clusters with the same two isolates (Figure 5B), also with moderate UFbootstrap support (UFBoot of 81%). The S segment clusters separately, between JCV and Inkoo virus, with weak UFbootstrap support of 60% (Figure 5C), suggesting its position in this phylogeny is variable.

In addition to JCV, via CPE assays on C7/10 cell-lines, we also isolated various insect-specific viruses (ISVs) presented in Table 2. Two Culex-flaviviruses were isolated in different mosquito pools (MP55; *Culex restuans* and MP62; *Aedes japonicus*), Houston virus and Phasi Charoen-like phasivirus were found in MP333 (*Culex territans*), and Phasi Charoen-like phasivirus was also found in MP323 (*Anopheles crucians*).

Discussion

We conducted a broader mosquito survey, considering an elevational and eco-region gradient, to address the lack of data on mosquito and arboviral diversity within the Commonwealth of Virginia. Our study demonstrates that Virginia has a high diversity of both mosquitoes and arboviruses. The

highest diversity of both viruses and mosquitoes, was found at Cavalier WMA on the coastal flood plains of the state.

Mosquitoes

At Cavalier WMA, we found multiple species of concern including *Culex coronator* and *Culex nigripalpus* (a new species based on range expansion). *Culex coronator* is a known vector for viruses like WNV (Mackay, Roy, Yates, & Foil, 2008), SLEV (Aitken, Downs, Spence, & Jonkers, 1964) and Zika Virus (ZIKV) (Elizondo-Quiroga et al., 2018). *Culex nigripalpus* is known to contribute to the WNV transmission cycle (Vitek, Richards, Mores, Day, & Lord, 2014) and has also been linked to the transmission of SLEV (Day & Curtis, 1993) and EEEV (Corrin, Ackford, Mascarenhas, Greig, & Waddell, 2021), all which are (re-)emerging arboviruses of concern to human health. The first record of *Culex coronator* mosquitoes in Virginia was in 2016, with subsequent collections in 2019 and 2020, in Suffolk County (Akaratovic & Kiser, 2017). A single female *Culex nigripalpus* was found in Virginia in 2017 in Suffolk County, Virginia (Akaratovic, Kiser, Whitt, Harrison, & Harrison, 2021). Our study is the second record of both of these species and the first record of either species outside of Suffolk County (i.e., neighboring Chesapeake County). Our study demonstrates the continuing range expansion of both species, showing an increased risk for pathogen transmission.

The two most common species of mosquito found were *Culiseta inornata* and *Culex pipiens* (Table 2). Both species are associated with human pathogenic arboviruses. *Culiseta inornata* has been associated with the transmission of viruses like CVV (Corner, Robertson, Hayles, & Iversen, 1980), California encephalitis virus (CEV) (Morgante & Shemanchuk, 1967), and JCV (Shepard & Armstrong, 2023). *Culex pipiens* is known to transmit a number of pathogens including WNV, Rift Valley fever virus, Sindbis virus, and heartworm (Dohm, Sardelis, & Turell,

2002; Meegan, Khalil, Hoogstraal, & Adham, 1980; Michalski, Erickson, Bartholomay, & Christensen, 2010; Molaei, Andreadis, Armstrong, Anderson, & Vossbrinck, 2006; Reeves, Hammon, & Izumi, 1942; Taylor, Hurlbut, Work, Kingston, & Frothingham, 1955). The presence of medically important mosquito species like *Culiseta inornata*, *Culex pipiens*, *Culex coronator*, and *Culex nigripalpus*, in a highly populated part of the Commonwealth heightens the need to increase surveillance efforts in the region.

Insect-specific viruses

In our study, we isolated various insect-specific viruses (ISV) (Table 3). I obtained two isolates of *Culex flavivirus* (CxFV) via MP55 (*Culex restuans*) and MP62 (*Aedes japonicus*). CxFV is an ISV discovered in Japan in the early 2000s (Hoshino et al., 2007). Since its initial discovery variants of CxFV have been found throughout the world (Blitvich et al., 2009; Cook et al., 2009; Farfan-Ale et al., 2009; Kim et al., 2009; Morales-Betoulle et al., 2008). Although ISVs are less intensely studied than vertebrate infectious viruses, a case can be made for their importance in disease transmission. A study conducted in Chicago demonstrated that mosquitoes that were positive for CxFV were about four times more likely to test positive for WNV (Newman et al., 2011). These studies go against the idea of superinfection exclusion as the infection with CxFV seems to increase the likelihood of infection with an arbovirus such as WNV (Newman et al., 2011). A similar pattern has been demonstrated for another of our isolates, Phasi Charoen-like phasi virus (PCLV) (MP323, *Anopheles crucians*; MP333, *Culex territans*). Previous studies on *Aedes aegypti* mosquitoes demonstrated that infection with PCLV and another ISV, Humaita Tubiacanga virus increases the likelihood of both dengue and Zika virus infections and transmission two-fold (Olmo et al., 2023). These studies demonstrate the importance of baseline knowledge of arboviruses,

including ISVs, as they could play a role in exacerbating the prevalence of other pathogenic viruses.

Another set of ISVs isolated are Houston virus and Nam Dinh virus (MP333; *Culex territans*), both belonging to the new viral family *Mesoniviridae* (Table 3) (Lauber et al., 2012). Houston virus was first isolated from a *Culex quinquefasciatus* mosquito in Yucatan, Mexico in 2017 and is considered a variant of Nam Dinh virus (Cigarroa-Toledo et al., 2018). Nam Dinh virus was also first isolated from a *Culex quinquefasciatus* in China in 2011 (Thuy et al., 2013). Viruses in the family *Mesoniviridae* are widespread and abundant worldwide and are regularly found during mosquito screenings (Vasilakis et al., 2014). However, no pathology has been associated with either of these viruses to date (Thuy et al., 2013). Further, laboratory studies should be conducted to assess if the patterns observed with CxFV and PCLV also hold for Houston and Nam Dinh virus.

Vertebrate virus

Jamestown Canyon virus is an emerging arbovirus in North America, and here was isolated from a pool of *Anopheles crucians* mosquitoes from Cavalier WMA (Table 3). Serological evidence of JCV has been observed in Virginia four decades ago (Clark, Crabbs, Watts, & Bailey, 1986). However, no JCV mosquito isolates have been reported in Virginia since its detection in the DELMARVA region (Delaware, Maryland, Virginia peninsula) for nearly five decades (Le Duc, Suyemoto, Eldridge, Russell, & Barr, 1975). Our study provides the first mosquito isolate of JCV in Virginia since the initial survey was conducted in the 1970s (Le Duc et al., 1975). This is a critical finding because, over the last 10 years, there has been a significant increase in reporting of JCV across the US, particularly in the Midwest and in New England (Pastula et al., 2015; Pastula

et al., 2016; Shepard & Armstrong, 2023). Our phylogenetic studies of the strain isolated show that both the L segment and M segment have moderate UFBootstrap support for the branches (Figure 5A-B), and the segments genetically cluster with JCV isolates from Massachusetts and Connecticut. The S segment however, has weak UFBootstrap support, suggesting that it can be an ancestral lineage of either JCV or Inkoo virus (European strain of JCV) (Figure 5C). However, further analysis will be required to determine if this is a new strain of JCV.

Several Rhabdovirus isolates were obtained, including Muir Springs virus (MP796; *Aedes canadensis*) and Flanders hapavirus (MP912; *Culiseta inornata*) (Table 3). Although I was able to identify MP764 as a Rhabdovirus (Table 3), its closest match on NCBI was Muir Springs virus, at only a 67% match, implying that this is possibly an undescribed virus. Nevertheless, our sequences were very patchy, and many segments were missing. Because of this "incomplete" sequence, further analysis is needed to confirm that our isolate is a new virus. The viral isolate from MP912 was also identified as a Rhabdovirus (Flanders hapavirus) (FLAV). FLAV is commonly found in ornithophilic mosquitoes and in avian hosts along the east coast (Golnar et al., 2018; McLean & Ubico, 2007; Whitney, 1964).

We found high viral diversity across three distinct eco-regions in Virginia, including viruses in five families, multiple insect-specific viruses, and, to the best of my knowledge, the first isolate of JCV since 1971. Our study also demonstrated the broad diversity of mosquitoes that occur in Virginia and the first account of two significant mosquito vectors (*Culex nigripalpus* and *Culex coronator*) in Chesapeake County, Virginia. My research confirms that more mosquito-borne viruses are expected to be detected with the increase of surveillance efforts, including human pathogenic viruses.

Acknowledgements

I would like to thank the field volunteers that assisted with field set up, including Xavier Garcia, Dyess Harp, Shaan Smith, and Lauren Panny. We would like to thank the Wildlife Management Area representatives, Ali Davis, Evan Sheer, and the staff at Big Survey WMA for making access to the field sites very manageable.

Funding

This work was partially supported by an integrated seed grant from the Center for Advanced Innovation in Agriculture, College of Agriculture and Life Sciences at Virginia Tech. This work was also partially supported by a grant from National Institute of Allergy and Infectious Diseases of the National Institutes of Health under award number U19AI181968 to AJA. KL was supported by a Gilliam Fellowship for Advanced Study from the Howard Hughes Medical Institute. MT was supported by a George Washington Carver Fellowship from the College of Agriculture and Life Sciences at Virginia Tech. This work was also partially supported by a contract from the United States Department of Agriculture Agricultural Research Service under agreement # 58-3022-4-044 to AJA. AJA was supported by the United States Department of Agriculture National Institute of Food and Agriculture, Hatch VA-160103, project 1020026. Figure schematics were created using Biorender.com. LEE was supported by National Science Foundation CAREER (2235295) and HEGS (2116748) awards, and Virginia Tech DA PPP, CeZAP, and ICTAS grants, and by the Chinese Academy of Sciences PIFI project 2024PVC0085. Research reported in this publication was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under Award Number K01AI168452. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National institutes of Health.

Disclosure Statement

No potential conflict of interest was reported by the author(s).

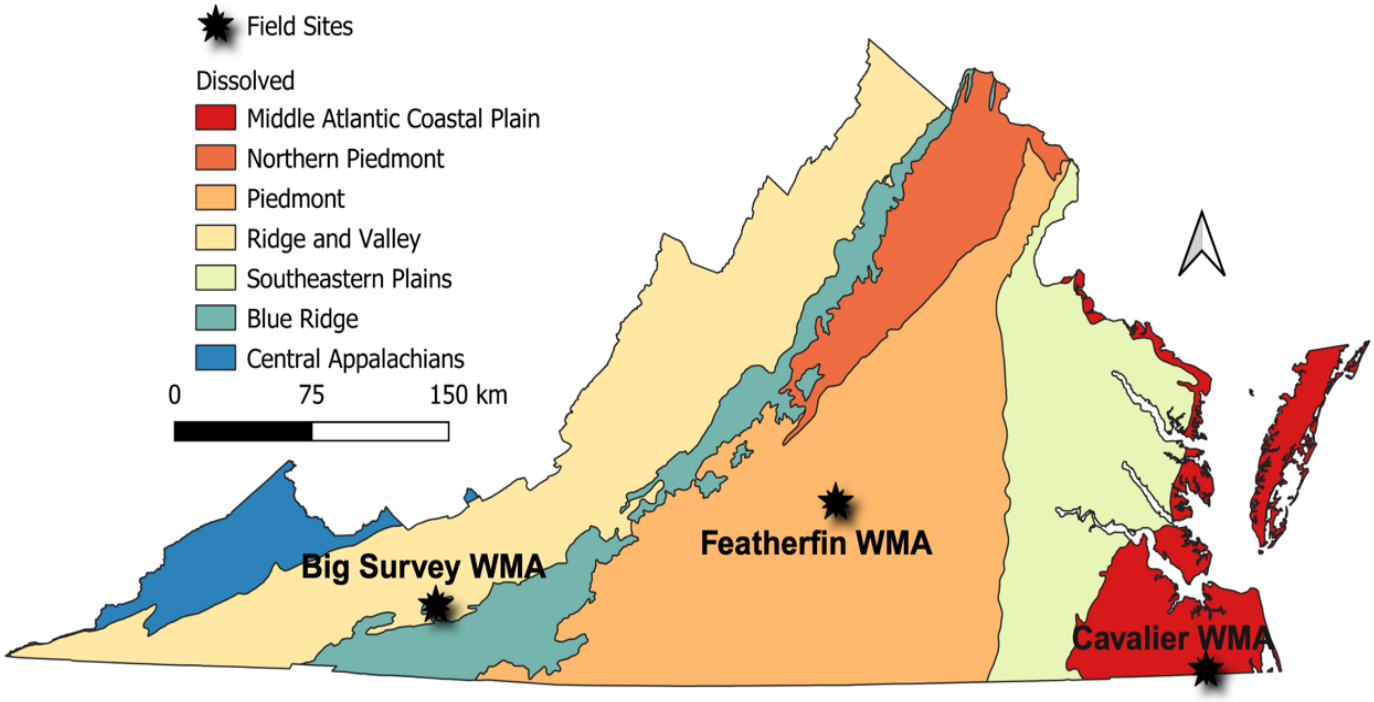


Figure 1. Map of the field sites used in the study. As well as the major ecological regions within the state of Virginia. Cavalier Wildlife Management Area (WMA) is located in Chesapeake County, Virginia with an elevation of 7 meters. The landscape features for Cavalier WMA include a combination of swamp, coastal flood plains and agriculture/farming. Featherfin WMA is in three counties Appomattox, Prince Edward and Buckingham counties. The landscapes featured in Featherfin include mixed prairie and Hardwood Forest edge with an elevation of 145 meters. Big Survey WMA is located in Wythe County and is composed of deciduous forest habitat with an elevation of 945 meters.

Figure 2. A mock set up of our Mosquito trap array. Every field site had four pairs of a CDC light trap baited with CO_2 and a gravid trap baited with maple leaf infused water. Trap pairs were strategically placed >100 meters away from each other and were in locations that we believed would have high mosquito potential (i.e., in shaded quiet spot). Image created using biorender.com

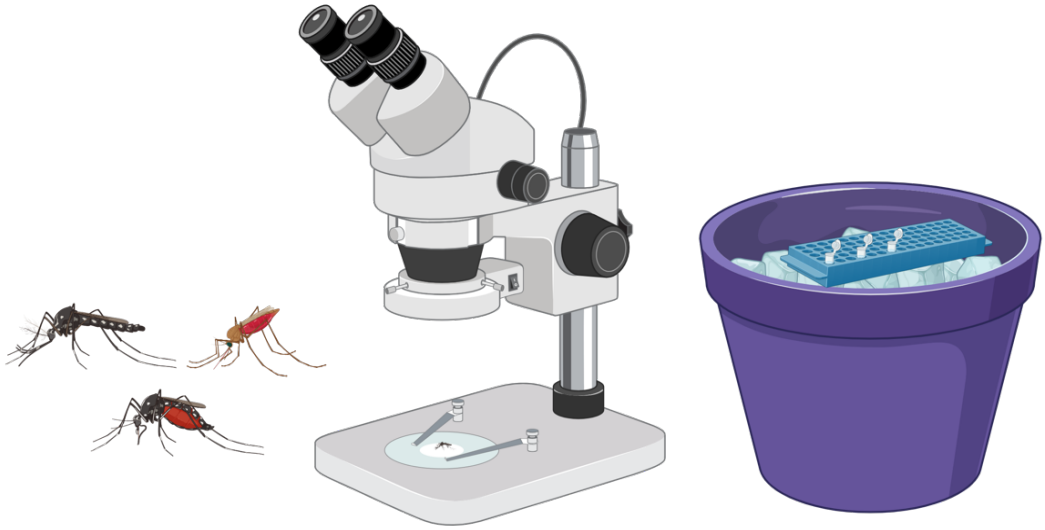


Figure 3. Mosquito identification/sorting was conducted using a dissecting scope and all mosquitoes were sorted into pools, separated by species, location, date and trap type (either CO_2-CDC baited light traps or Gravid traps). All sorting was conducted on dry ice to preserve any potential viruses in the samples. Image created using biorender.com

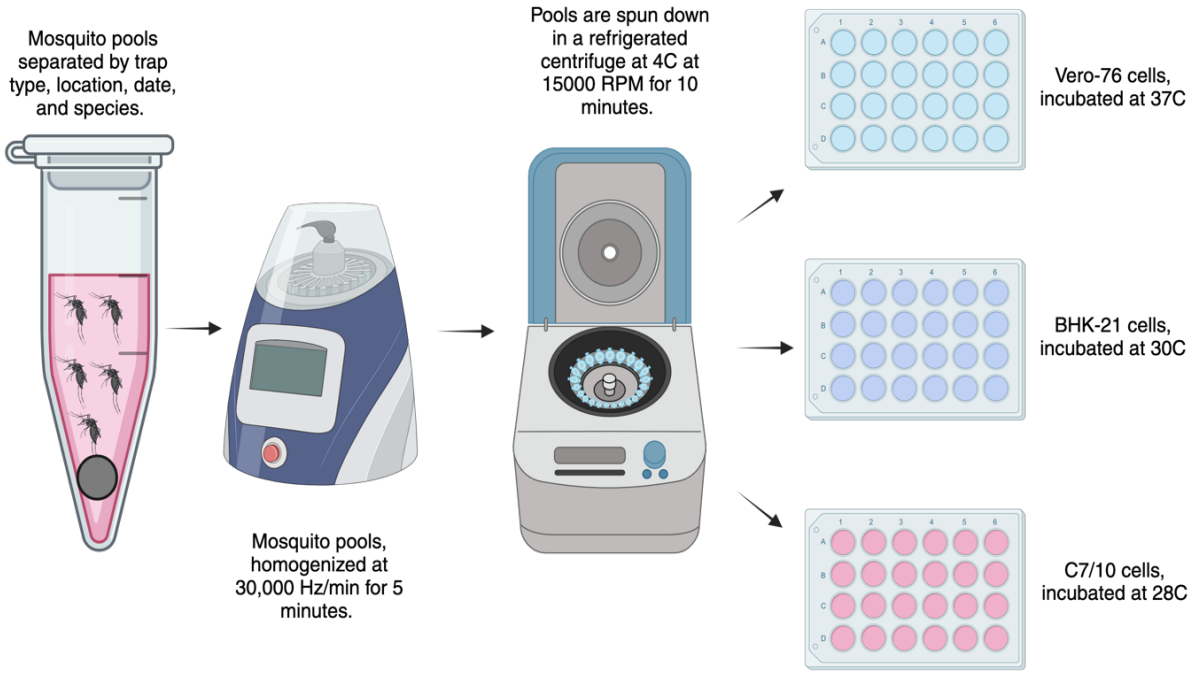


Figure 4a. Schematic of initial viral screening. Mosquitoes were sorted into pools, and these pools were homogenized at 30,000 Hz/min for 5 minutes. The samples were then clarified using a refrigerated centrifuge at 4ºC at a speed of 15000 RMP for 10 minutes, this step was conducted twice. The samples were then plated in three distinct cell lines, Vero-76 at 37 ºC, BHK-21 at 30 ºC, and C7/10 at 28 ºC. The samples were monitored daily for 14 days and harvested upon observation of cytopathic effect.

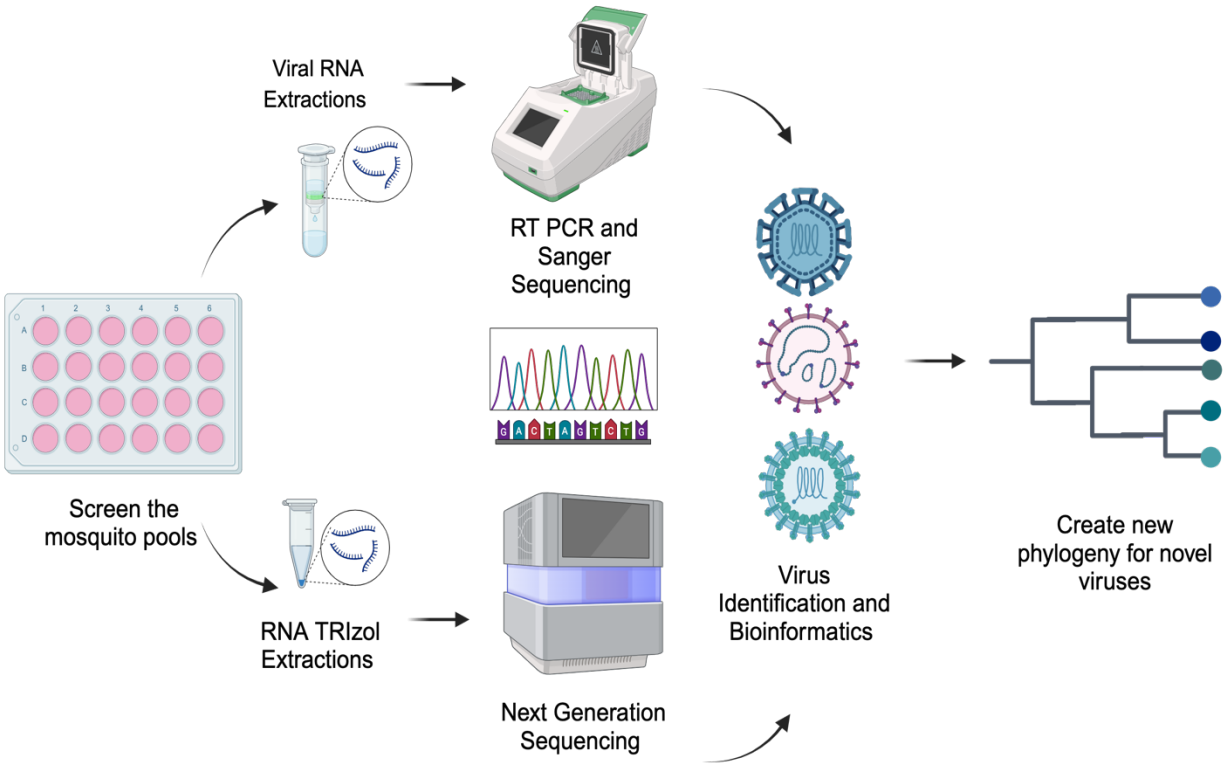


Figure 4b. CPE-positive samples were RNA extracted either via viral RNA extraction kits (Qiagen) for Sanger sequencing or TRIzol LS for Next Generation Sequencing (NGS) for viral identification. The RT-PCR were run using three different primer sets, Pan-Alphavirus, Pan-Flavivirus, or Pan-Bunyavirus. Samples that presented with strong bands were extracted and sent for Sanger sequencing. Samples that presented with cytopathic effect, but did not react to our primers, were TRIzol extracted and sent off for NGS. Upon, receiving the raw data, samples were cleaned (low quality) and trimmed, aligned, host genomes removed. The identified viral reads were assembled by *de novo* assemblers using Megahit and ABySS 2.0 to generate longer sequences. Alignment of contigs and unassembled reads to viral reference genomes was performed using Geneious Prime 2021.0.7 (https://www.geneious.com).

Table 1. Sequences for primers used to conduct diagnostic PCRs for virus screening

Primer	Forward	Reverse	Citation
Pan-Alphavirus	NSP1-F-M2W 5'-YAGAGCDTTTTCGCAYSTRGCHW -3'	NSP1-R-cM3W 5'-ACATRAANKGNGTNGTRTCRAANCCDAYCC -3'	Bronzoni et al., 2005
Pan-Flavivirus	NS5 -F-FG1 5'-TCAAGGAACTCCACACATGAGATGTACT -3'	NS5-R-FG2 5'-GTGTCCCATGCTTCATCAGCATACA -3'	Bronzoni et al., 2005
Pan-Bunyavirus	BUN-S+ F 5'-AGTAGTGTACTCCAC -3'	BUN-S-R 5'-AGTAGTGTGCTCCAC -3'	Dunn, Pritlove, & Elliott, 1994

Table 2. Mosquitoes found in Virginia Survey at the three field sites including Big Survey WMA, Cavalier WMA, and Featherfin WMA.

Species	Big Survey	Cavalier	Featherfin	Total
Aedes albopictus	0	1 (1)	10 (9)	11 (10)
Aedes atlanticus/tormentor	0	29 (9)	0	29 (9)
Aedes canadensis	1 (1)	34 (8)	0	35 (9)
Aedes cantator	0	2 (1)	0	2 (1)
Aedes cinereus	0	2 (2)	0	2 (2)
Aedes fulvus palens	0	1 (1)	0	1 (1)
Aedes infirmatus	0	2 (2)	2 (1)	4 (3)
Aedes japonicus	59 (25)	0	3 (3)	62 (28)
Aedes sollicitans	0	7 (1)	0	7 (1)
Aedes sticticus	3 (2)	12 (8)	0	15 (10)
Aedes taeniorhynchus	0	13 (3)	0	13 (3)
Aedes triseriatus	76 (35)	21 (16)	7 (6)	104 (57)
Aedes trivittatus	0	3 (2)	0	3 (2)
Aedes vexans	2 (2)	179 (21)	62 (13)	243 (36)
Anopheles barberi	10 (8)	0	0	10 (8)
Anopheles crucians	0	192 (49)	0	192 (49)
Anopheles punctipennis	1 (1)	32 (17)	49 (20)	82 (38)
Anopheles quadrimaculatus	0	75 (31)	0	75 (31)
Anopheles walkeri	0	15 (7)	0	15 (7)
Coquillettidia perturbans	1 (1)	292 (37)	5 (4)	298 (42)
Culex coranator	0	3 (3)	0	3 (3)
Culex erraticus	0	242 (46)	6 (3)	248 (49)
Culex nigripalpus	0	2 (2)	0	2 (2)
Culex pipiens/quinquefasciatus	0	1472 (104)	29 (8)	1501 (112)
Culex restuans	38 (16)	98 (21)	284 (26)	420 (63)
Culex salinarius	0	6 (2)	0	6 (2)
Culex territans	3 (3)	22 (17)	4 (4)	29 (24)
Culiseta inornata	1 (1)	2730 (185)	0	2731 (186)
Culiseta melanura	0	62 (7)	0	62 (7)
Orthopodomyia spp.	6 (6)	1 (1)	0	7 (7)
Psorophora ciliata	0	0	1 (1)	1 (1)
Psorophora columbiae	0	1 (1)	0	1 (1)
Psorophora ferox	0	1 (1)	1 (1)	2 (2)
Uranotaenia sapphirina	0	1030 (62)	1 (1)	1031 (63)
Total	201 (101)	6582 (666)	464 (100)	7247 (867)

Numbers represent the actual total mosquitoes found at each site, (#) represent the number of pools.

Table 3. Viruses found in Virginia from screened mosquito pools.

Virus Family	Virus Species	No. of Isolates	Mosquito pool ID and species	County Location for each isolate
Peribunyaviridae	Jamestown Canyon virus	1	MP564; *Anopheles crucians*	Chesapeake
Flaviviridae	Culex flavivirus	2	MP55; *Culex restuans*	Montgomery
			MP62; *Aedes japonicus*	
Mesoniviridae	Nam Dinh virus	1	MP333; *Culex territans*	Chesapeake
	Houston virus			
Rhabdoviridae	Muir Springs virus	1	MP796; *Aedes canadensis*	Chesapeake
	Flanders hapavirus	1	MP912; *Culiseta inornata*	Chesapeake
Phenuiviridae	Phasi Charoen-like phasivirus	2	MP323; *Anopheles crucians*	Chesapeake
			MP333; *Culex territans*	Chesapeake

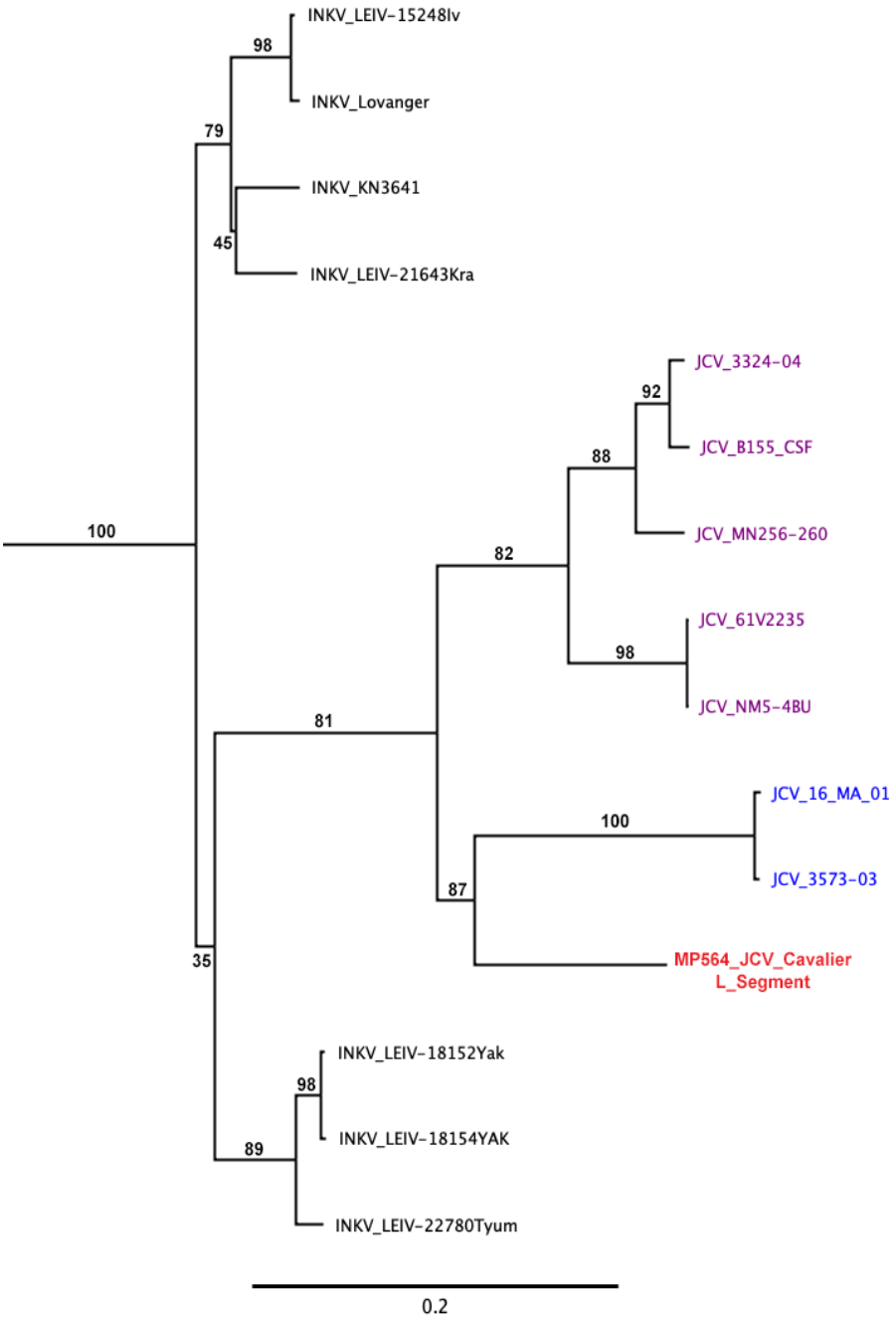


Figure 5a. Phylogenetic analysis of the L segment for Jamestown Canyon virus isolate (MP564, *Anopheles crucians*; Cavalier WMA). Our data show, that the L segment clusters with the JCV isolates from Massachusetts (JCV-16_MA-01) and Connecticut (JCV_3573-03) with moderate UFbootstrap support (UFBoot of 87%). Analysis for the trees were run using Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-aLRT) and Ultrafast Bootstrap (UFBoot) in the program IQ-tree. The trees were rooted using an outgroup LACV_77 (L Segment Accession #

DQ196118.1), and LACV_HAY539; (L Segment Accession # OP594812.1) the outgroups were removed for clarity purposes. Figtree was used to visualize and edit the trees.

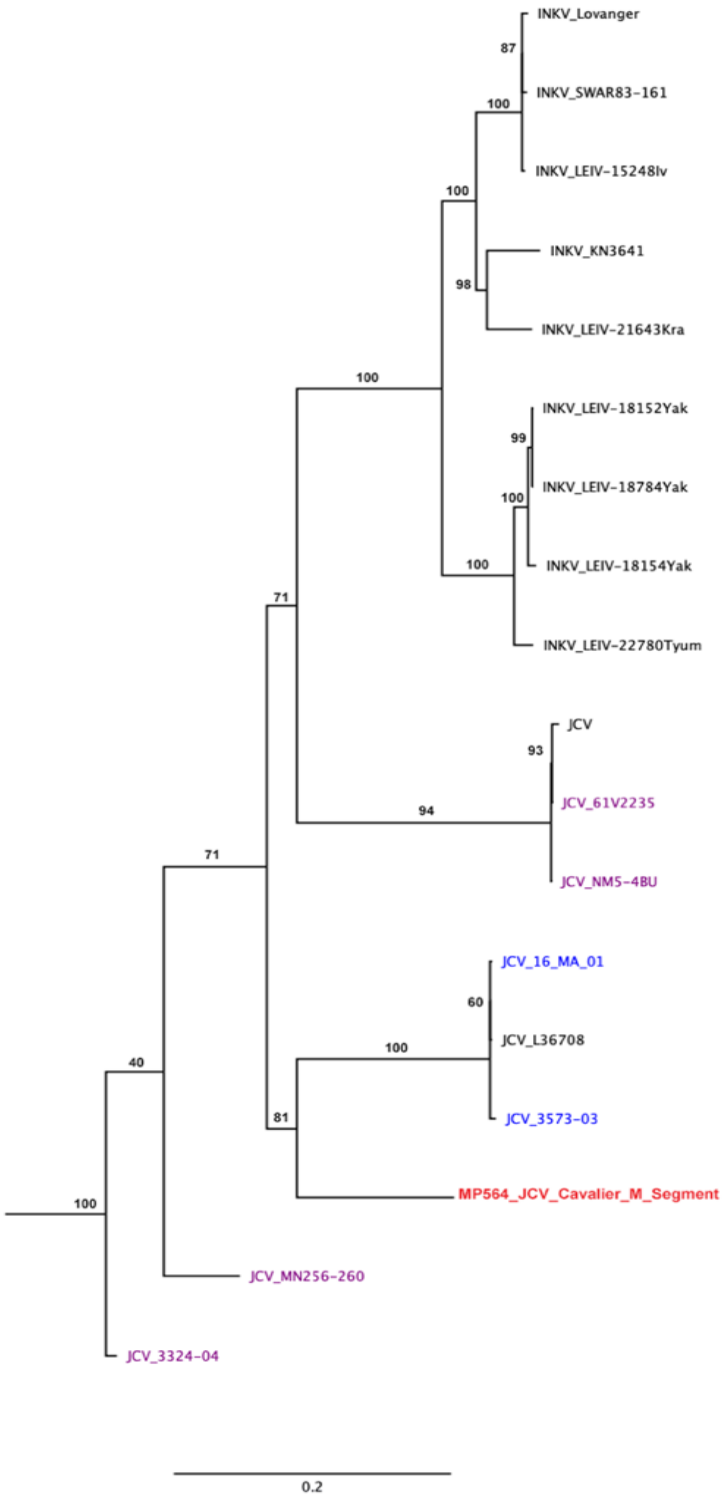


Figure 5b. Phylogenetic analysis of the M segment for Jamestown Canyon virus isolate (MP564, *Anopheles crucians*; Cavalier WMA). The M segment, clusters with JCV isolates from

Massachusetts (JCV-16_MA-01) and Connecticut (JCV_3573-03) with moderate UFbootstrap support of 81%. Analysis for the trees were run using Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-aLRT) and Ultrafast Bootstrap (UFBoot) in the program IQ-tree. The trees were rooted using an outgroup LACV_77 (M segment Accession # DQ196119.1) and LACV_HAY539; (M segment Accession # OP594811.1) the outgroups were removed for clarity purposes. Figtree was used to visualize and edit the trees.

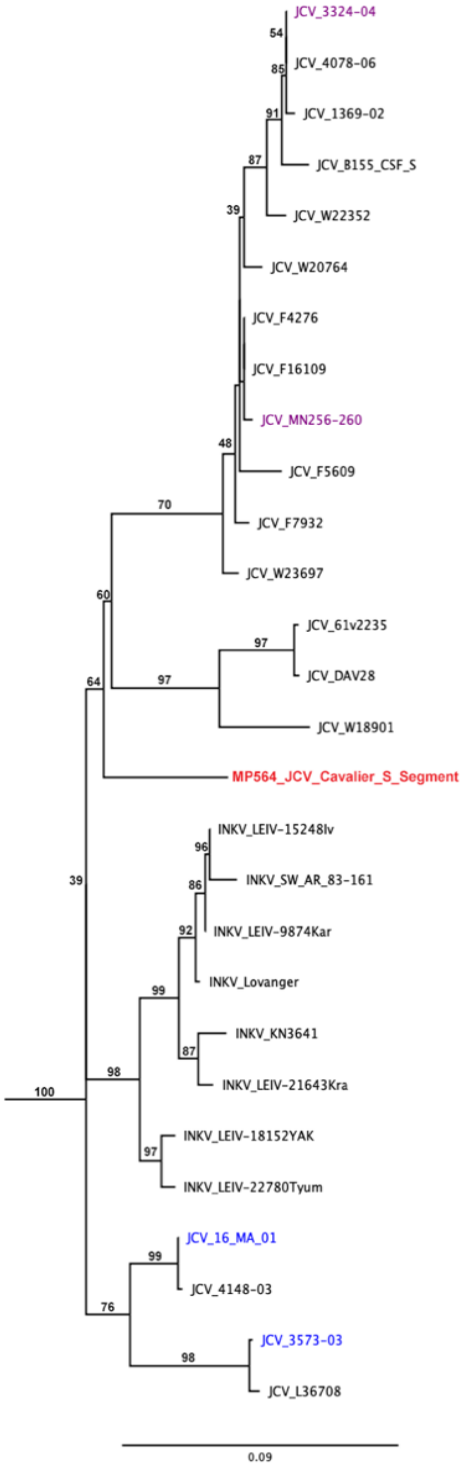


Figure 5c. Phylogenetic analysis of the S segment for Jamestown Canyon virus isolate (MP564, *Anopheles crucians*; Cavalier WMA). The S segment branches out on its own, in between JCV and Inkoo virus, with a weak UFbootstrap support of only 60%. Analysis for the trees were run

using Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-aLRT) and Ultrafast Bootstrap (UFBoot) in the program IQ-tree. The trees were rooted using an outgroup LACV_77 (S segment Accession # DQ196120.1) and LACV_HAY539; (S segment Accession # OP594810.1) the outgroups were removed for clarity purposes. Figtree was used to visualize and edit the trees.

References

Aitken, T. H., Downs, W. G., Spence, L. P., & Jonkers, A. H. (1964). St Louis Encephalitis Virus Isolations in Trinidad, West Indies, 1953-1962. *Am J Trop Med Hyg*, 450-451.

Akaratovic, K. I., & Kiser, J. P. (2017). First record of *Culex coronator* in Virginia, with notes on its rapid dispersal, trapping methods, and biology. *Journal of the American Mosquito Control Association, 33*(3), 225-228.

Akaratovic, K. I., Kiser, J. P., Whitt, P. B., Harrison, R. L., & Harrison, B. A. (2021). *Culex nigripalpus* distribution expansion: first record in Virginia, New County records in North Carolina, and revised United States map. *Journal of the American Mosquito Control Association, 37*(4), 188-197.

Andrews, S. (2010). FastQC: a quality control tool for high throughput sequence data. Cambridge, United Kingdom.

Blitvich, B. J., Lin, M., Dorman, K. S., Soto, V., Hovav, E., Tucker, B. J., . . . Bartholomay, L. C. (2009). Genomic sequence and phylogenetic analysis of *Culex flavivirus*, an insect-specific flavivirus, isolated from *Culex pipiens* (Diptera: Culicidae) in Iowa. *Journal of Medical Entomology, 46*(4), 934-941.

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics, 30*(15), 2114-2120.

Bronzoni, R. V. d. M., Baleotti, F. G., Ribeiro Nogueira, R. M., Nunes, M., & Moraes Figueiredo, L. T. (2005). Duplex reverse transcription-PCR followed by nested PCR assays for detection and identification of Brazilian *alphaviruses* and *flaviviruses*. *Journal of clinical microbiology, 43*(2), 696-702.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. L. (2009). BLAST+: architecture and applications. *BMC bioinformatics, 10*, 1-9.

Cigarroa-Toledo, N., Baak-Baak, C. M., Cetina-Trejo, R. C., Cordova-Fletes, C., Martinez-Nuñez, M. A., Talavera-Aguilar, L. G., . . . Blitvich, B. J. (2018). Complete genome sequence of houston virus, a newly discovered mosquito-specific virus isolated from *Culex quinquefasciatus* in Mexico. *Microbiology Resource Announcements, 7*(10),10-1128.

Clark, G. G., Crabbs, C., Watts, D. M., & Bailey, C. L. (1986). An ecological study of Jamestown Canyon virus on the Delmarva Peninsula, with emphasis on its possible vector. *Journal of Medical Entomology, 23*(6), 588-599.

Cohen, J. M., Smith, D. L., Cotter, C., Ward, A., Yamey, G., Sabot, O. J., & Moonen, B. (2012). Malaria resurgence: a systematic review and assessment of its causes. *Malaria journal, 11*, 1-17.

Cook, S., Moureau, G., Harbach, R. E., Mukwaya, L., Goodger, K., Ssenfuka, F., . . . de Lamballerie, X. (2009). Isolation of a novel species of flavivirus and a new strain of Culex flavivirus (*Flaviviridae*) from a natural mosquito population in Uganda. *Journal of General Virology, 90*(11), 2669-2678.

Corner, L. C., Robertson, A. K., Hayles, L. B., & Iversen, J. O. (1980). Cache Valley virus: experimental infection in *Culiseta inornata*. *Canadian journal of microbiology, 26*(3), 287-290.

Corrin, T., Ackford, R., Mascarenhas, M., Greig, J., & Waddell, L. A. (2021). Eastern equine encephalitis virus: a scoping review of the global evidence. *Vector-Borne and Zoonotic Diseases, 21*(5), 305-320.

Darsie, R., Jr, & Ward, R. A. (2005). *Identification and geographical distribution of the mosquitoes of North America,North of Mexico*. Univerity Press of Florida, Gainesville, FL, USA

Day, J., & Curtis, G. (1993). Annual emergence patterns of *Culex nigripalpus* females before, during and after a widespread St. Louis encephalitis epidemic in south Florida. *Journal of the American Mosquito Control Association, 9*(3), 249-255.

Dohm, D. J., Sardelis, M. R., & Turell, M. J. (2002). Experimental vertical transmission of West Nile virus by *Culex pipiens* (Diptera: Culicidae). *Journal of Medical Entomology, 39*(4), 640-644.

Dunn, E. F., Pritlove, D. C., & Elliott, R. M. (1994). The s rna genome segments of batai, cache valley, guaroa, kairi, lumbo, main drain and northway bunyaviruses: Sequence determination and analysis. *Journal of General Virology, 75*(3), 597-608.

Elizondo-Quiroga, D., Medina-Sánchez, A., Sánchez-González, J. M., Eckert, K. A., Villalobos-Sánchez, E., Navarro-Zúñiga, A. R., . . . Arias, C. F. (2018). Zika virus in salivary glands of five different species of wild-caught mosquitoes from Mexico. *Scientific reports, 8*(1), 809.

Farfan-Ale, J. A., Loroño-Pino, M. A., Garcia-Rejon, J. E., Hovav, E., Powers, A. M., Lin, M., . . . Soto, V. (2009). Detection of RNA from a novel West Nile-like virus and high prevalence of an insect-specific flavivirus in mosquitoes in the Yucatan Peninsula of Mexico. *The American journal of tropical medicine and hygiene, 80*(1), 85.

Foster, W. A., & Walker, E. D. (2019). Mosquitoes (Culicidae). *Medical and Veterinary Entomology* (Vol. 3rd Edition pp. 261-317). Academic Press, Cambridge, MA, USA

Golnar, A. J., Langevin, S., Panella, N. A., Solberg, O. D., Reisen, W. K., & Komar, N. (2018). Flanders hapavirus in western North America. *Archives of virology, 163*, 3351-3356.

Hoshino, K., Isawa, H., Tsuda, Y., Yano, K., Sasaki, T., Yuda, M., . . . Sawabe, K. (2007). Genetic characterization of a new insect flavivirus isolated from *Culex pipiens* mosquito in Japan. *Virology, 359*(2), 405-414.

Jackman, S. D., Vandervalk, B. P., Mohamadi, H., Chu, J., Yeo, S., Hammond, S. A., . . . Warren, R. L. (2017). ABySS 2.0: resource-efficient assembly of large genomes using a Bloom filter. *Genome research, 27*(5), 768-777.

Kim, D. Y., Guzman, H., Bueno Jr, R., Dennett, J. A., Auguste, A. J., Carrington, C. V., . . . Tesh, R. B. (2009). Characterization of Culex Flavivirus (*Flaviviridae*) strains isolated from mosquitoes in the United States and Trinidad. *Virology, 386*(1), 154-159.

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature methods, 9*(4), 357-359.

Lauber, C., Ziebuhr, J., Junglen, S., Drosten, C., Zirkel, F., Nga, P. T., . . . Gorbalenya, A. E. (2012). *Mesoniviridae*: a proposed new family in the order Nidovirales formed by a single species of mosquito-borne viruses. *Archives of virology, 157*, 1623-1628.

Le Duc, J. W., Suyemoto, W., Eldridge, B. F., Russell, P. K., & Barr, A. (1975). Ecology of California encephalitis viruses on the Del Mar Va Peninsula. II. Demonstration of trans-ovarial transmission. *The American journal of tropical medicine and hygiene, 24*(1), 124-126.

Li, D., Liu, C.-M., Luo, R., Sadakane, K., & Lam, T.-W. (2015). MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics, 31*(10), 1674-1676.

Liu, N. (2015). Insecticide resistance in mosquitoes: impact, mechanisms, and research directions. *Annual review of entomology, 60*(1), 537-559.

Loftin, K. C., Herbert, M. W., & Phaltankar, P. G. (2006). Five-year surveillance of West Nile and eastern equine encephalitis viruses in southeastern Virginia. *Journal of environmental health, 68*(9), 33.

Lwande, O. W., Obanda, V., Lindström, A., Ahlm, C., Evander, M., Näslund, J., & Bucht, G. (2020). Globe-trotting *Aedes aegypti* and *Aedes albopictus*: risk factors for arbovirus pandemics. *Vector-Borne and Zoonotic Diseases, 20*(2), 71-81.

Mackay, A. J., Roy, A., Yates, M. M., & Foil, L. D. (2008). West Nile virus detection in mosquitoes in East Baton Rouge parish, Louisiana, from November 2002 to October 2004. *Journal of the American Mosquito Control Association, 24*(1), 28-35.

Madewell, Z. J. (2020). Arboviruses and their vectors. *Southern Medical Journal, 113*(10), 520.

McLean, R. G., & Ubico, S. R. (2007). Arboviruses in birds. *Infectious diseases of wild birds*, 17-62.

Meegan, J. M., Khalil, G. M., Hoogstraal, H., & Adham, F. K. (1980). Experimental transmission and field isolation studies implicating *Culex pipiens* as a vector of Rift Valley fever virus in Egypt. *The American journal of tropical medicine and hygiene, 29*(6), 1405-1410.

Metz, S. W., & Pijlman, G. P. (2011). Arbovirus vaccines; opportunities for the baculovirus-insect cell expression system. *Journal of Invertebrate Pathology, 107*, S16-S30.

Michalski, M. L., Erickson, S. M., Bartholomay, L. C., & Christensen, B. M. (2010). Midgut barrier imparts selective resistance to filarial worm infection in *Culex pipiens pipiens*. *PLoS Neglected tropical diseases, 4*(11), e875.

Minh, B. Q., Nguyen, M. A. T., & von Haeseler, A. (2013). Ultrafast Approximation for Phylogenetic Bootstrap. *Molecular Biology and Evolution, 30*(5), 1188-1195.

Molaei, G., Andreadis, T. G., Armstrong, P. M., Anderson, J. F., & Vossbrinck, C. R. (2006). Host feeding patterns of *Culex* mosquitoes and West Nile virus transmission, northeastern United States. *Emerging Infectious Diseases, 12*(3), 468.

Morales-Betoulle, M., Pineda, M. M., Sosa, S., Panella, N., Cordon-Rosales, C., Komar, N., . . . Johnson, B. (2008). Culex flavivirus isolates from mosquitoes in Guatemala. *Journal of Medical Entomology, 45*(6), 1187-1190.

Morgante, O., & Shemanchuk, J. A. (1967). Virus of the California encephalitis complex: isolation from *Culiseta inornata*. *Science, 157*(3789), 692-693.

Musso, D., Rodriguez-Morales, A. J., Levi, J. E., Cao-Lormeau, V.-M., & Gubler, D. J. (2018). Unexpected outbreaks of arbovirus infections: lessons learned from the Pacific and tropical America. *The Lancet Infectious Diseases, 18*(11), e355-e361.

Newman, C. M., Cerutti, F., Anderson, T. K., Hamer, G. L., Walker, E. D., Kitron, U. D., . . . Goldberg, T. L. (2011). *Culex flavivirus* and West Nile virus mosquito coinfection and positive ecological association in Chicago, United States. *Vector-Borne and Zoonotic Diseases, 11*(8), 1099-1105.

Nguyen, L.-T., Schmidt, H. A., von Haeseler, A., & Minh, B. Q. (2014). IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. *Molecular Biology and Evolution, 32*(1), 268-274.

Olmo, R. P., Todjro, Y. M., Aguiar, E. R., de Almeida, J. P. P., Ferreira, F. V., Armache, J. N., . . . Silva, A. T. S. (2023). Mosquito vector competence for dengue is modulated by insect-specific viruses. *Nature microbiology, 8*(1), 135-149.

Pastula, D., Johnson, D. H., White, J., Dupuis, A., Fischer, M., & Staples, J. (2015). Jamestown Canyon virus disease in the United States—2000–2013. *The American journal of tropical medicine and hygiene, 93*(2), 384.

Pastula, D., Smith, D., Beckham, J., & Tyler, K. (2016). Four emerging arboviral diseases in North America: Jamestown Canyon, Powassan, chikungunya, and Zika virus diseases. *J Neurovirol, 22*(3), 257-260.

Perrin, A., Glaizot, O., & Christe, P. (2022). Worldwide impacts of landscape anthropization on mosquito abundance and diversity: A meta-analysis. *Global Change Biology, 28*(23), 6857-6871.

Reeves, W. C., McD. Hammon, W., & Izumi, E. M. (1942). Experimental Transmission of St. Louis Encephalitis Virus by *Culex pipiens* Linnaeus. *Proceedings of the Society for Experimental Biology and Medicine, 50*(1), 125-128.

Rochlin, I., Ninivaggi, D., Hutchinson, M., & Farajollahi, A. (2013). Climate change and range expansion of the Asian tiger mosquito (*Aedes albopictus*) in Northeastern USA: implications for public health practitioners. *PLoS One, 8*(4), e60874.

Romero-Alvarez, D., Escobar, L. E., Auguste, A. J., Del Valle, S. Y., & Manore, C. A. (2023). Transmission risk of Oropouche fever across the Americas. *Infectious diseases of poverty, 12*(1), 47.

Schwab, S. R., Stone, C. M., Fonseca, D. M., & Fefferman, N. H. (2018). The importance of being urgent: the impact of surveillance target and scale on mosquito-borne disease control. *Epidemics, 23*, 55-63.

Shepard, J. J., & Armstrong, P. M. (2023). Jamestown Canyon virus comes into view: understanding the threat from an underrecognized arbovirus. *Journal of Medical Entomology, 60*(6), 1242-1251.

Soper, F. L. (1963). The elimination of urban yellow fever in the Americas through the eradication of *Aedes aegypti*. *American Journal of Public Health and the Nations Health, 53*(1), 7-16.

Taylor, R., Hurlbut, H., Work, T., Kingston, J., & Frothingham, T. (1955). Sindbis Virus: a newly recognized Arthropod transmitted Virus. *The American journal of tropical medicine and hygiene, 4*(5), 844-862.

Thuy, N. T., Huy, T. Q., Nga, P. T., Morita, K., Dunia, I., & Benedetti, L. (2013). A new nidovirus (NamDinh virus NDiV): Its ultrastructural characterization in the C6/36 mosquito cell line. *Virology, 444*(1-2), 337-342.

Vasilakis, N., Guzman, H., Firth, C., Forrester, N. L., Widen, S. G., Wood, T. G., . . . Blasdell, K. R. (2014). Mesoniviruses are mosquito-specific viruses with extensive geographic distribution and host range. *Virology journal, 11*, 1-12.

Virginia Department of Health. (2019). *VDH Monthly Mosquito Activity Report* Retrieved from https://www.vdh.virginia.gov/?s=VDH+monthly+mosquito+activity+report#gsc.tab=0&gsc.q=VDH%20monthly%20mosquito%20activity%20report&gsc.page=1, Accessed on December 26, 2024.

Virginia Department of Wildlife Resources, V. (2024a). Big Survey Wildlife Management Area. Retrieved from https://dwr.virginia.gov/wma/big-survey/, Accessed on December 26, 2024.

Virginia Department of Wildlife Resources, V. (2024b). Cavalier Wildlife Management Area. Retrieved from https://dwr.virginia.gov/wma/cavalier/, Accessed on December 26, 2024.

Virginia Department of Wildlife Resources, V. (2024c). Featherfin Wildlife Management Area. Retrieved from https://dwr.virginia.gov/wma/featherfin/, Accessed on December 26, 2024.

Virginia Museum of History & Culture. (2024). The Regions of Virginia Retrieved from https://virginiahistory.org/learn/regions-virginia, Accessed on December 26, 2024.

Vitek, C. J., Richards, S. L., Mores, C. N., Day, J. F., & Lord, C. C. (2014). Arbovirus transmission by *Culex nigripalpus* in Florida, 2005. *Journal of Medical Entomology, 45*(3), 483-493.

Whitney, E. (1964). Flanders Strain, an Arbovirus newly isolated from Mosquitoes and Birds of New York State. *The American journal of tropical medicine and hygiene, 13*(1), 123-131.

Wilson, A. L., Courtenay, O., Kelly-Hope, L. A., Scott, T. W., Takken, W., Torr, S. J., & Lindsay, S. W. (2020). The importance of vector control for the control and elimination of vector-borne diseases. *PLoS Neglected tropical diseases, 14*(1), e0007831.

Conclusions

My doctoral work focused on the development of animal models and mosquito surveillance for Orthobunyaviruses. I explored this by developing a murine model for Cache Valley virus (CVV), a neglected *Orthobunyavirus* in North America (Chapter 1). I also looked at the potential role birds could play in the transmission of CVV and conducted both *in vitro* and *in vivo* studies in various poultry species (Chapter 2). Lastly, I wanted to explore the ecology of Orthobunyaviruses, mosquitoes and their distribution within the Commonwealth of Virginia by conducting vector and arboviral surveillance surveys (Chapter 3).

In Chapter 1 (López et al. 2021), I explored the pathogenesis of CVV by developing a murine model that replicates the natural progression of disease observed in ruminants. Prior to this work, there were no available small animal models to study CVV pathogenesis, which significantly limited the scope of research that could be conducted and severely hindered the development of therapeutics for the virus. My research shows that CVV disease in mice is dependent on innate immune responses and that type 1 interferon signaling is necessary to prevent infection in mice. $IFNAR^{-/-}$ mice inoculated with a lethal dose of CVV presented with significant disease in all the age groups studied. However, the immune competent models (C57BL/6 and CD-1 mice) demonstrated age dependent pathogenesis; the younger mice succumbed to disease, whereas the adults showed no morbidity or mortality. I also developed an *in-utero* transmission model, this study showed high rates of transmission, spontaneous abortions, and congenital malformation in the offspring. CVV infection in our model presented with high tissue tropism in the liver, spleen, and reproductive organs. Considering the extremely high rates of seropositivity in ruminants across the US, the expansion of competent disease vectors, the potential for epizootic emergence of CVV is extremely high. The development of this animal model will provide researchers with a novel

tool to explore both short- and long- term pathogenesis for CVV and provide a good platform to test and validate vaccines and therapeutics for this emerging pathogen. Future research should focus on the development of an immune competent models, consider mouse adapted viral strains, and explore the influence of various inoculation routes that mimic a natural infection (i.e., similar to that of a mosquito bite).

For Chapter 2, I explored the pathogenesis and growth kinetics of CVV within poultry species. Due to CVV's extensive geographic range, reports of birds being seropositive for the closely related viruses, Cholul or Maguari, and the fact that CVV has been isolated from highly ornithophilic mosquito vectors, there is evidence that implies the virus may infect poultry species. For both our *in-vitro* and *in-vivo* studies, I employed two strains of the two known genetic lineages of CVV to assess its potential as a disease-causing agent in poultry species. I evaluated the growth kinetics of CVV in three avian cell lines: Pekin duck cells (*Anas platyrhynchos domesticus*: PDE), Japanese quail (*Coturnix coturnix japonica*: QNR/K2), and domestic chicken (*Gallus gallus*; DF-1). For *in-vivo* experiments I exposed three-day-old SPF-chickens (*Gallus gallus*), three-day-old ducklings (*Anas platyrhynchos domesticus*), and fourteen-day-old quail (*Coturnix coturnix*). I discovered that although CVV expanded quickly and to high titers in all three avian cell lines *in-vitro*, it was unable to cause a noticeable infection *in-vivo*. According to my research, domestic poultry species probably do not play a big role in the maintenance and transmission of CVV. To find out if CVV can affect bird species, more research utilizing passerines and mosquito transmission studies is required. It would also be useful to repeat this study with the two closely related viruses (Cholul and Maguari), to determine if those viruses do cause disease in living poultry.

For Chapter 3, I attempted to look at the diversity of viruses and mosquito vectors for the state of Virginia. Establishing a recognized baseline of pathogens and vectors is essential to creating effective disease outbreak management plans as the effects of climate change and the range expansion of viable disease vectors become more apparent. The natural landscape of Virginia is varied, ranging from mountain ranges to coastal wetlands. By surveying across the different regions, we can explore how variations in landscape characteristics might affect the spread of vectored pathogens. For one week each month from May through October 2022, I conducted surveys at three locations around Virginia: Piedmont (savannah), Coastal Plain (swamp), and Blue Ridge Mountains (primary forest). CO_2-baited CDC light and gravid traps were used to gather mosquitoes, which were then grouped into pools according to species, location, date, and trap type. Mosquitoes totaling 7,247 individuals were gathered and divided into 867 pools. Cytopathic effect (CPE) assays were used to identify pools with potential viruses and the diagnostic PCRs using Pan-Flavi-, pan-Bunya-, and pan-Alpha-virus primers were employed to identify virus families, and pathogens were identified by sequencing amplicons. I found 58 insect-specific viruses, including two Culex Flaviviruses, Nam Dinh virus, Houston virus, and Phasi Charoen-like phasivirus, and three vertebrate viruses including two Rhabdoviruses with unknown pathology (Flanders hapavirus and Muir Springs virus), and one strain of an encephalitic vertebrate virus (i.e., Jamestown Canyon virus; an orthobunyavirus). In addition to identifying vertebrate viruses of concern, our survey confirmed the first detection of two invasive mosquito species (*Culex nigripalpus* and *Culex coronator)* in Chesapeake County, VA. In addition to helping public health officials create risk assessments for Virginia and its neighboring states, this study provides insight into the vast undiscovered viral diversity that exists throughout the state of Virginia.

Orthobunyaviruses are among the most diverse groups of viruses yet are some of the least studied. Although understudied, orthobunyaviruses still contribute significantly to the burden of disease, in some cases leading to encephalitis and even death. CVV is incredibly widespread in North America yet continues to be a severely understudied pathogen. My dissertation provided the first ever successful small animal models for studying CVV pathogenesis. I plan on using these models in the future for the validation and testing of various novel CVV vaccines and therapeutics. My research also provided further insights on the role birds might play in the amplification of CVV in agricultural settings. Future studies will focus on using a recombinant strain of CVV and Potosi virus (Cholul virus) and Maguari virus to identify if these closely related viruses can cause disease in avian species. Lastly, my work provided invaluable evidence of vector expansion for two important disease vectors (*Culex coronator* and *Culex nigripalpus*) into a highly populated county of Virginia. We also identified many insect-specific arboviruses, and isolated another strain of Jamestown Canyon virus, an emerging orthobunyavirus in North America of significant public health concern. I believe this work provides critical information to Public Health bodies in the Commonwealth of Virginia including, regarding how they can adjust their current arboviral surveillance program, for example, by including orthobunyaviruses into their panel of mosquito testing. In addition to this research providing a base for my future work; I hope that my work insights the development of further research efforts with orthobunyaviruses and surveillance studies.