Improving flavivirus prevention and control measures by assessing the protection of swine by anti-Japanese encephalitis virus monoclonal antibodies and determining North American mosquito competence for Usutu virus

by

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B.S., Oklahoma State University, 2015
M.S., Texas A&M University, 2017

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
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Abstract

Flaviviruses cause diseases of critical human and veterinary public health importance. The Japanese encephalitis (JE) serocomplex includes flaviviruses transmitted by mosquito vectors. Within this serocomplex, Usutu virus (USUV) has had a severe impact on the European black bird population, has caused several cases of neurological disease in humans, and has been reported to co-circulate with West Nile virus (WNV). With JE serocomplex viruses currently circulating in North America (WNV and St. Louis encephalitis virus) it is critical to determine competent species that could establish an enzootic transmission cycle and contribute to flavivirus maintenance outside endemic regions.

The determination of vector competence can help assess the likelihood that flaviviruses of the JE serocomplex such as USUV can establish their transmission cycle in North America. In this work, it was demonstrated that two common North American mosquito species, *Culex pipiens* and *Culex quinquefasciatus*, are susceptible to USUV and competent for its transmission should this disease be introduced to North America. In contrast, North American *Aedes albopictus* were found to be refractory to infection with USUV, suggesting the species would be unlikely to contribute to USUV transmission in the New World.

While vector surveillance and control are important, it is essential to continually evaluate, improve, and create vaccines against flaviviruses. This includes JE virus (JEV), which causes an estimated 68,000 cases of JE annually in humans, with a subset of these infections resulting in severe and chronic neurological complications. Existing vaccines are derived from only one of the five JEV genotypes, leading to concerns of reduced cross-protection, and necessitating further investigation into prevention measures. Current *in vivo* models used to assess JE vaccines are limited due to their dissimilarity to human physiology. As swine are natural amplification hosts

of JEV and experience disease outcomes similar to human infection, swine could prove beneficial over current models in characterizing humoral immunity and evaluating the protection provided by vaccination derived monoclonal antibodies (mAbs). Miniature swine were utilized to evaluate prophylactic passive immunization using mAbs to assess protection against an emerging JEV genotype. Since JE vaccine-induced immune responses can target the envelope (E) protein, vaccination derived IgG mAbs reactive with different antigenic structures in the E protein, JEV-31 (E domain III) and JEV-169 (E domain I and domain II), were selected. Results indicated that the passive immunization of either mAb reduced severity of disease including fever, viremia, viral shedding, systemic infection, and neuroinvasion. Overall, this work aims to improve flavivirus prevention and control measures by increasing our understanding of vector species that should be targeted for control and assessing a swine-based *in vivo* platform to better investigate flavivirus prevention measures.

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Dedication

To Boomer and Kody.

Chapter 1 - Literature Review

Overview of flaviviruses

The *Flaviviridae* family is a large group of viruses that have been associated with human disease for centuries. Flavivirus infections are believed to have occurred as early as the mid-1600s with descriptions of what is thought to be yellow fever (Tomori, 2004). The family was named after the Latin word "flavus" meaning "yellow" due to the jaundice that can occur during infection. In subsequent years flaviviruses have continued to cause diseases across the world necessitating the need for improvements to prevention measures.

The *Flavivirdae* family contains 89 species in four genera: *Flavivirus*, *Hepacivirus*, *Pegivirus*, and *Pestivirus* (ICTV, 2021). The *Flavivirus* genus can be further sub-categorized into four major groups, insect specific flaviviruses (ISFV), no known vector flaviviruses (NKVF), tick-borne flaviviruses (TBFV), and mosquito-borne flaviviruses (MBFV) (Fig. 1-1). This dissertation will provide details on viruses within the Japanese encephalitis (JE) serocomplex of the *Flaviviridae* family with a focus on Usutu virus (USUV) and Japanese encephalitis virus (JEV). The JE serocomplex is further discussed on page 11.

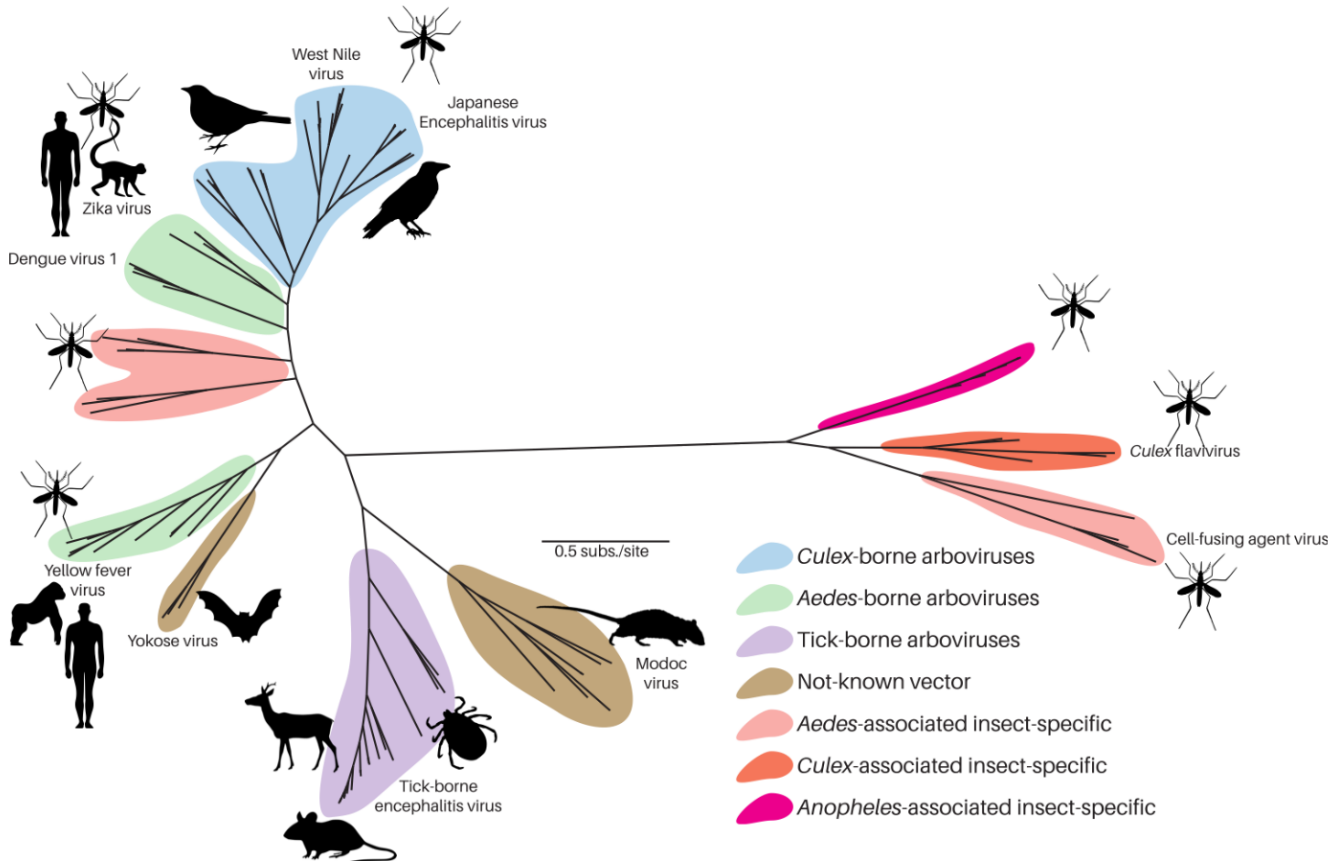

Figure 1-1. Phylogeny of the Flavivirus genus with host type and virus representatives. Published under the Creative Commons (Lequime, 2020).

Insect specific flaviviruses are generally referred to as viruses that naturally infect hematophagous *Diptera*, replicate in mosquito cells, but do not replicate or infect vertebrates (Bolling et al., 2015). Over thirty ISFV have been described and they are separated into two distinct groups depending on their phylogenetic and antigenic relationships (Blitvich and Firth, 2015; Guzman et al., 2018; Moureau et al., 2010). The largest group is the classical ISFV, and the second group is known as the dual host ISFV (Blitvich and Firth, 2015; Guzman et al., 2018).

The NKVF, sometimes referred to as vertebrate-specific flaviviruses, can also be further separated into two groups. This includes those isolated from rodents (e.g.: Modoc virus) and those

isolated from bats (e.g.: Rio Bravo virus). It has been suggested that these viruses are maintained via horizontal transmission among hosts (Adams et al., 2013; Blitvich and Firth, 2015; Burns and Farinacci, 1956; Fairbrother and Yuill, 1987).

The TBFV can cause severe disease manifestations including infection of the central nervous system with up to 30% lethality (Barbic et al., 2013; Gould and Solomon, 2008; Kemenesi and Bányai, 2018; LaSala and Holbrook, 2010). Some of the tick-transmitted flaviviruses include Kyasanur Forest disease virus (KFDV), Omsk hemorrhagic fever virus, Powassan virus, and tick-borne encephalitis virus (TBEV). The TBFV consist of the tick-borne encephalitis serocomplex with two clades named mammalian and seabird TBFV to indicate differences in the primary vertebrate host (Gould and Solomon, 2008; LaSala and Holbrook, 2010). Transmission of TBFV can occur through the saliva of infected ticks as well as transmitted among tick populations by feeding on the same host. Transmission can also occur transovarially (from infected females to their eggs) and trans-stadially (from one development stage to the next), with an infected tick remaining infected for life (Danielová et al., 2002; Labuda et al., 1993a; Labuda et al., 1997; Labuda et al., 1993b). While human infection can occur following a tick bite, other routes of TBFV transmission through the consumption of unpasteurized milk or milk products from infected livestock have been described (Brockmann et al., 2018; Hudopisk et al., 2013; Offerdahl et al., 2016; Paulsen et al., 2019). The initial phase of tick-borne infection in humans can present with non-specific symptoms such as headache and fatigue, while the secondary phase can range in presentation from mild meningitis to severe encephalitis (Bogovic and Strle, 2015; Kaiser, 2008; Lindquist and Vapalahti, 2008). The severe disease symptoms and spread to new geographic locations make TBFV an emerging threat to humans.

The MBFV can cause severe morbidity and mortality worldwide (Gould et al., 2017; Gubler, 1998; Huang et al., 2019). They continue to be global health concerns as globalization, urbanization, and climate change have expanded the geographic location of both hosts and vectors (Ashraf et al., 2015; Devaux, 2012; Gao et al., 2019; Gould et al., 2017; Le Flohic et al., 2013). A wide variety of mosquito species are capable of transmitting MBFV, with *Aedes* spp. mosquitoes primarily associated with hemorrhagic flaviviruses such as dengue virus (DENV) and yellow fever virus (YFV). *Culex* spp. are often linked to the transmission of encephalitic flaviviruses including JE serocomplex members. Investigation of mosquitoes of both *Aedes* and *Culex* spp. for their competence of MBFV will be important in improving flavivirus prevention measures. The viruses within the MBFV group vary in their ability to infect humans and animals and differ in disease symptoms and pathogenesis which will also play a role in establishing risk assessments and mitigation strategies.

Flaviviridae genomic and structural characteristics

Genomic characteristics

Flaviviridae are spherical viruses 40-60 nanometers in diameter that contain a positive single stranded RNA genome (ICTV, 2021). The genome of *Flaviviridae* is between 11-12 kilobases (kb) with a 5′ cap and no poly A tail. The genome is translated as a single open reading frame (ORF) into one polyprotein. This protein is modified during and after translation to create three structural and seven nonstructural proteins.

Structural and nonstructural proteins

The three structural proteins are the capsid (C), pre-membrane (prM), and envelope (E) protein. They are encoded in the ORF downstream of the 5′ noncoding region of the genome. The

C protein is involved in packaging the viral genome, with multiple copies of the C protein binding single copies of viral RNA and forming the nucleocapsid (Mukhopadhyay et al., 2005). In immature virions, the prM protein aids in folding an assembly of the E protein (Unni et al., 2011). The E protein plays a vital role in the viral attachment, membrane fusion, and entry of the virus into the host's cells and will be further discussed in the last subsection of this heading (Kulkarni et al., 2017). The seven nonstructural (NS) proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 are essential for viral replication (Chambers et al., 1990).

The envelope protein

The flavivirus E protein is an ~53 kilodalton elongated protein composed predominantly of β-strands and possesses three distinct domains that are connected by flexible hinge regions (Kulkarni et al., 2017; Luca et al., 2012; Mukhopadhyay et al., 2005). The three domains of the E protein are domain I (EDI), domain II (EDII), and domain III (EDIII). The EDI is an eight-stranded β barrel located at the center of the E protein, while EDII consists of two long finger-like structures with a highly conserved fusion loop that is responsible for the membrane fusion during viral entry. The EDIII has an immunoglobulin-like structure at the C-terminus of the E protein that is thought to be a receptor binding region (Sun et al., 2017).

Arthropod-borne virus transmission

Arthropod-borne viruses or arboviruses are viruses that are maintained in nature primarily through biological transmission between susceptible vertebrate hosts by blood feeding arthropods. Biological transmission involves an arthropod becoming infected with a virus and the virus replicating in the vector. The extrinsic incubation period is the time between the vector imbibing a viremic bloodmeal to the time that the virus can be transmitted by the vector to a new host. In

the vertebrate host, the period from infection to disease is called the intrinsic incubation period (Higgs and Beaty, 2005).

Transmission of these viruses can be vertical from vector to offspring, or horizontal from vector to host. Vertical transmission of arboviruses can occur through transovarial transmission or transovum transmission to infect progeny (Tesh et al., 2016). In addition to vector to host, horizontal transmission between male and female vectors can occur by venereal transmission during copulation or by non-replicative transmission from co-feeding on a nonviremic host (Higgs and Beaty, 2005). Other methods of transmission include the transmission of virus from arthropod vector to host through nonbiological transmission routes, either direct or mechanical transmission. Direct transmission of an arbovirus can occur through contact between hosts, or animals becoming infected after eating mosquitoes that are infected with the virus (Kuno and Chang, 2005). Mechanical transmission is when a vector transfers virus from its contaminated mouthparts when switching hosts during feeding, without the vector being infected itself (Kuno and Chang, 2005).

The transmission of mosquito-borne flaviviruses depends on the presence of competent vectors and susceptible hosts, as well as the capacity for transmission. The competence of a vector is its intrinsic ability to biologically transmit a disease agent (Higgs & Beaty, 2005). Vector competence studies are able to evaluate this by observing infection, dissemination, and transmission. The capacity of a vector to transmit disease is dependent on the vector competence, density of vectors to vertebrate hosts, feeding frequency, and the extrinsic incubation period (Macdonald, 1961). The focus of this dissertation is on mosquito-borne flaviviruses, specifically two that are primarily transmitted by *Culex* species mosquitoes.

Flavivirus vaccines

Currently there ae no specific antivirals available against flaviviruses, therefore vaccination is an attractive and effective approach to reduce the burden of disease. Licensed human vaccines against flaviviruses exist for DENV, JEV, KFDV, TBEV, and YFV. Currently licensed vaccines against flavivirus for humans are summarized in Table 1-1, with the exception of JE vaccines which will be discussed in the JE vaccines and treatments section. The commercially available flavivirus vaccines will be discussed briefly due to their importance in reducing flavivirus disease and their potential to help create additional flavivirus vaccines in the future.

Table 1-1. Current human licensed flavivirus vaccines except Japanese encephalitis.

Virus	Vaccine Type	Manufacturer	Availability
Dengue	Live-attenuated	Sanofi Pasteur	Asia and Latin America
Kyasanur Forest disease	Inactivated	Institute of Animal Health and Veterinary Biologicals (Bangalore, Karnataka)	India
Tick-borne encephalitis	Inactivated	Baxter AG (Europe), Microgen (Russia), CIBP (China)	China, Europe, Russia
Yellow fever	Live-attenuated	Worldwide including Sanofi Pasteur	Worldwide

Inactivated vaccine platforms eliminate the possibility of viral replication, which can be beneficial for vaccinating immunocompromised populations who are particularly susceptible to flavivirus infection. There are currently licensed inactivated vaccines against KFDV and TBEV

(Kubinski et al., 2020). A formalin inactivated vaccine against KFDV was produced in chicken embryo fibroblasts and has been used since 1990 in India (Thomas et al., 2015). The vaccine requires two doses one month apart with boosters after six to nine months (Thomas et al., 2015).

The formalin inactivated TBE vaccines contain strains of European or Far-Eastern TBEV. The virus isolates for European and Russian TBE vaccines are grown in primary chicken embryonic cells and the Chinese TBE vaccine is grown in primary hamster kidney cells (Kubinski et al., 2020). Two doses of the TBE vaccines are given between two and three months apart followed by a booster one year after the second dose (Barrett, 2001). Additional boosters of the TBE vaccine are recommended every three years (Barrett, 2001).

Live-attenuated vaccines have the advantage of eliciting a rapid and robust immune response, generally requiring a single dose rather than multiple boosters. Live-attenuated platforms exist for flaviviruses, including the YF-17D vaccine and Dengvaxia®. The YF-17D vaccine was developed in 1937 and is considered to be one of the most successful vaccines, with one dose conferring long term immunity (Barrett and Teuwen, 2009). The YF-17D vaccine was obtained through the passage of the Asibi strain in chicken embryos 176 times to remove its neurotropic properties. Three 17D substrains (17D-204, 17DD, and 17D-213) are used as vaccines with minor differences in genome sequences, though all create efficacious vaccines (Beck and Barrett, 2015). Since its licensure over 500 million doses of the YF-17D vaccine have been administered worldwide (Barrett and Teuwen, 2009).

There is also a live-attenuated vaccine against DENV (Dengvaxia®), that was first licensed in 2016 and approved by the United States Food and Drug Administration in May 2019 (Thomas and Yoon, 2019). Dengvaxia® is a tetravalent vaccine consisting of chimeras made up of structural prM and E genes of the four DENV types combined with the nonstructural genes of the

yellow fever 17D vaccine strain. Two phase three efficacy trials were used to evaluate the vaccine and the current recommendation for Dengvaxia® is three doses given six months apart (Thomas and Yoon, 2019).

Vaccines against flaviviruses have been demonstrated to be effective. Live-attenuated vaccines against flaviviruses provide greater immunity due to replication of virus in the vaccine; however, the risk of reversion keeps them from typically being used in immunocompromised individuals. The inactivated vaccines against TBE and KFD have also been successful, though they require multiple booster immunizations to maintain immunity.

Overview of monoclonal antibodies

Antibodies can provide protection against flavivirus infection. Antibodies are Y-shaped proteins produced by the immune system when it detects a foreign substance called an antigen. The specific regions of the antigen recognized by the antibody are called antigenic determinants or epitopes. Flavivirus vaccines introduce the antigen to stimulate an immune response to produce antibodies. This means that if that if a flavivirus enters the body it can be recognized by a B cell, and that B cell will produce antibodies. The antibodies can react with discontinuous residues brought together by folding, or be continuous; recognizing a linear stretch of amino acids (Nelson et al., 2000). Antigens present many different epitopes; therefore, polyclonal antibodies can be produced which are antibodies from different B cell clones that recognize various epitopes. There are also antibodies with unique specificity, derived from a single B cell clone, and specific for a single epitope that are called a monoclonal antibodies (mAbs) (Nelson et al., 2000). The antibodies discussed in this dissertation are mAbs, they have advantages over polyclonal antibodies due to their consistency, specificity, and reproducibility. These attributes aid in their use to investigate prophylactic and therapeutic options against flaviviruses.

The investigation of antibodies for therapeutic use began in 1975 when Kohler and Milstein described the *in vitro* production of murine mAbs from hybridomas (Kohler and Milstein, 1975). A hybridoma is a cell formed by the fusion of a short-lived antibody producing B cell and an immortal myeloma cell that expresses one specific antibody (Zaroff and Tan, 2019). The steps necessary for hybridoma generation and mAb selection are detailed in Fig. 1-2.

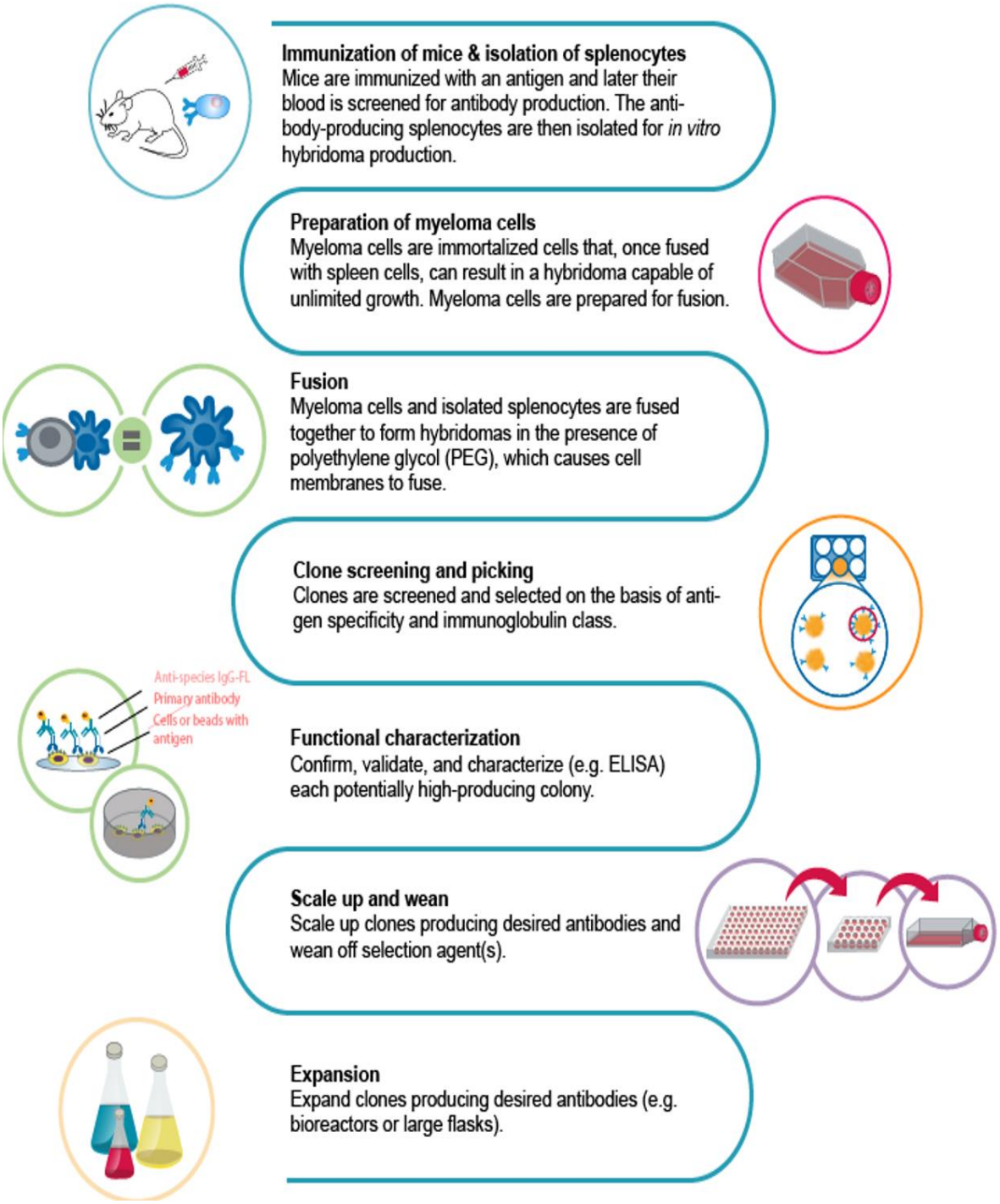

Figure 1-2. The steps of monoclonal antibody production. Image provided courtesy of Molecular Devices, LLC and used with permission (Molecular Devices, 2021). The generation of monoclonal antibodies includes immunizing a mouse with antigen, checking the antibody titer, and reimmunizing the mouse if the antibody titer is not sufficient. When sufficient antibody titer is reached, the mouse is euthanized, and the spleen removed. Myeloma cells are prepared by ensuring sensitivity to the hypoxanthine-aminopterin-thymidine (HAT) medium. This can be

achieved by culturing the myeloma cells in 8-azaguanine. Myeloma cells are immortal cancer cells and when fused with the spleen cells care capable of unlimited growth. Fusion of the myeloma cells and spleen cells occurs in polyethylene glycol, resulting in hybridomas. The cells are then grown in the HAT medium that only permits the growth of hybridoma cells. The medium containing the hybridoma cells is diluted into multi-well plates so that each well contains a single cell. Then screening for the presence of the desired antibody occurs (e.g., enzyme linked immunosorbent assay) and chosen clones are grown.

These steps have been utilized for the development of mAbs for therapeutic use, and led to the first mAb approved by the United States Food and Drug Administration, Orthoclone OKT3® (muromonab-CD3), in 1986 (Ecker et al., 2015). Given the concerns regarding the spread of infectious diseases across the world, the development and investigation of novel and efficacious interventions including mAbs is necessary.

Advances in technology and antibody engineering have made the development and marketing of mAbs possible. The range of diseases that have therapeutic mAbs in development and investigation has increased in the last three decades with more than 60 mAbs currently approved by the United States Food and Drug Administration. These mAbs target a wide variety of diseases and can provide support in the development and evaluation of vaccines for emerging infections (Boyiadzis and Foon, 2008; Tsumoto et al., 2019). This includes the assessment of vaccine efficacy and immunogenic epitopes, which could speed up the process of regulatory reviews. Furthermore, they can provide necessary information critical for vaccine formulation and safety profiles.

Antibodies against flaviviruses

The development of neutralizing antibodies is needed for robust immune responses against flaviviruses and selection of mAbs. The E protein is a primary target for neutralizing flaviviruses, and neutralizing epitopes have been defined within each of the three domains of the E protein. The regions of the E protein recognized by some of these epitopes are shown in Figure 1-3. The EDI

is the central domain containing 120 residues in three segments (1-51, 137-189, and 285-302). Antibodies against this region are often type specific, against a single virus serotype. Antibodies that target EDI have been specifically described against the EDI lateral ridge and the EDI-EDII hinge region. This includes the West Nile virus (WNV) EDI lateral ridge mAb E121, that exhibited protection in mice one and two days after infection (Oliphant et al., 2006). The WNV mAb 75G targets the EDI-EDII hinge region and was less protective against infection in mice than E121 after challenge (Oliphant et al., 2006). A chimpanzee DENV-4 neutralizing mAb, 5H2, is also reactive to EDI (Lai et al., 2007). The 5H2 mAb demonstrated the type specificity of EDI mAbs, neutralizing three strains of DENV serotype four and provided *in vivo* protection in monkeys against challenge (Lai et al., 2007).

The EDI-EDII hinge has been demonstrated to elicit type specific neutralizing antibodies. Antibody 1F4 against the EDI-EDII hinge is a human mAb that neutralizes DENV *in vitro* and *in vivo* (Fibriansah et al., 2014). There are also WNV mAbs against the EDI-EDII hinge, CR4354 and E113, with CR4354 conferring complete protection against challenge in mice (Oliphant et al., 2006; Vogt et al., 2009). The EDII consists of discontinuous residues 52-136 and 190-284 which includes the fusion loop at residues 98-110. The fusion loop is highly conserved across flaviviruses and because of this, antibodies generated against the EDII fusion loop are sometimes found to be cross-reactive with multiple flaviviruses. A mAb 2A10G6 was found to be reactive with the DENV fusion loop and was determined to also neutralize DENV1-4, YFV, and WNV *in vitro* (Deng et al., 2011). This suggests the fusion loop region may contain epitopes that are broadly neutralizing.

Potently neutralizing antibodies against flaviviruses have been mapped against the EDIII region. This domain includes around 100 continuous residues. Of particular interest is the EDIII

lateral ridge, made up of the BC loop (330-333), the DE loop (365-368), the FG loop (389-391) and the N-terminus liker region. Antibodies against EDIII are generally type specific, including WNV E16, recognizing the EDIII lateral ridge (Nybakken et al., 2005; Oliphant et al., 2005). Antibodies against EDIII have also been effective against DENV infection (Crill and Roehrig, 2001; Gromowski and Barrett, 2007). The protection demonstrated by mAbs against flaviviruses suggest that therapeutics against flaviviruses could be created in the future.

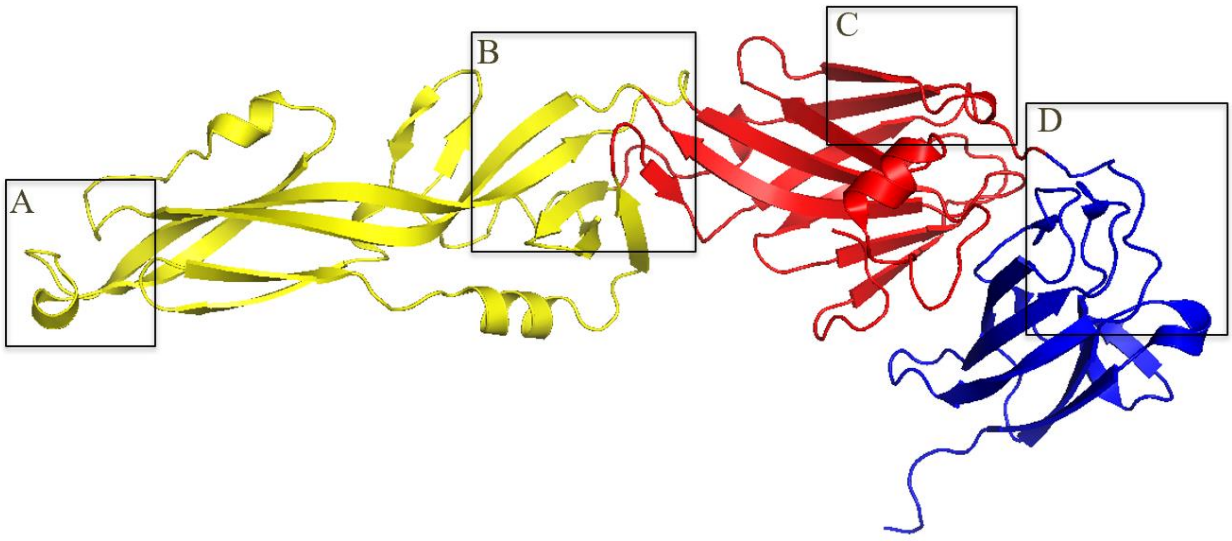

Figure 1-3. The crystal structure of the flavivirus envelope protein. Neutralizing antibodies against flaviviruses have been found to span all three domains of the envelope protein. The locations of neutralizing epitopes include A) the fusion loop (EDII), B) the EDI-EDII hinge, C) the EDI lateral ridge, and D) the EDIII lateral ridge. Domains I, II, and III are colored in red, yellow, and blue respectively (PDB access number: 3P54).

Animal research models for flaviviruses

Animal models including mice and nonhuman primates, have been utilized to investigate flavivirus pathogenesis as well as evaluate therapeutics and vaccines. Immunocompetent mice are generally resistant to flavivirus infection and disease, while mice with genetic deficiencies in type

I interferon are more susceptible (Rossi et al., 2016; Samuel and Diamond, 2005; Shresta et al., 2004). In studies of Zika virus (ZIKV), immunocompetent adult mice are not very susceptible to infection, while immune deficient mice display disease signs (Pena et al., 2018). While immunodeficient mice can develop viremia, they still do not always exhibit the same disease manifestations that humans with flavivirus infections experience. Additionally, as they lack components of the immune response there are limitations to their translation to human medicine. Similarly, with DENV, immunocompetent mice do not develop severe disease unless inoculated intracranially (Zompi and Harris, 2012).

Due to immunocompetent mice not demonstrating aspects of flavivirus disease in humans, nonhuman primates have also been used for evaluations of flaviviruses including rhesus and macaques. However, nonhuman primates can still be unable to manifest the same symptoms as humans and it is dependent on the route of challenge (Estes et al., 2018). In primates challenged with ZIKV, intracranial and intravenous challenge routes result in more severe outcomes than subcutaneous challenge (Morrison et al., 2017). Nonhuman primates to evaluate DENV immunopathogenesis also have drawbacks in their ability to develop clinical disease (Zompi and Harris, 2012). Limitations to nonhuman primate models are the high cost and availability. As would be expected, there are few laboratories with the ability to perform nonhuman primate experiments.

While animal models are essential to assess flavivirus vaccines, it can be difficult to develop a model able to adequately evaluate safety and efficacy for humans. For example, current models for studying flavivirus infection, tropism, and pathogenesis use challenge routes unable to emulate human disease. It is difficult to replicate the route of inoculation of natural mosquito-

borne flavivirus infection, which is introduced into the skin, as well as disease pathogenesis; therefore, the investigation of alternative flavivirus animal models is needed to address these gaps.

Japanese encephalitis serocomplex overview

Of the *Flavivirus* genus, the Japanese encephalitis (JE) serocomplex is comprised of eight antigenically related viruses including Cacipacore virus (CPCV), JEV, Koutango virus, Murray Valley encephalitis virus (MVEV), St. Louis encephalitis virus (SLEV), USUV, WNV, and Yaounde virus. Members of the JE serocomplex have recently emerged and become established in novel geographic regions. Historically JEV had been found in the Asia-Pacific region and has recently expanded to the Torres Strait of Australia in 1995 (Hanna et al., 1996). Geographic expansion also occurred with WNV, detected for the first time in the New World in 1999 when it caused an epidemic of encephalitis in New York City (Lanciotti et al., 2000). Since then WNV has become established in the United States, with 664 WNV disease cases reported and 505 classified as neuroinvasive as of May 2021 (CDC, 2021b). Another virus that has recently expanded its range is USUV, where in 2001 a massive decline in the blackbird population occurred in Austria and has since been detected in other European countries (Ashraf et al., 2015). Since flaviviruses are located on all continents with the exception of Antarctica, improving surveillance, prevention, and control programs is important for protecting human and animal health (Mackenzie et al., 2002; Mackenzie et al., 2004).

Japanese encephalitis serocomplex species-specific characteristics

The eight viruses of the JE serocomplex are transmitted by mosquito vectors, though they vary in host species and disease characteristics. While JEV and USUV will be described in detail

later in this dissertation, the other six members of the JE serocomplex will be briefly described to provide comparisons among this group (Table 1-2).

Table 1-2. Comparison of the Japanese encephalitis serocomplex.

Virus	Location	Vertebrate Hosts	Can cause human disease	Type of human disease
Cacipacore	South America	Bird	Yes	One patient with acute febrile illness
Japanese encephalitis	Asia, Australia	Bird, pig	Yes	Encephalitic
Koutango	Africa	Rodent	Yes*	Fever and rash
Murray Valley encephalitis	Australia, New Guinea	Bird	Yes	Encephalitic
St. Louis encephalitis	The Caribbean, North America, South America	Bird	Yes	Encephalitic
Usutu	Africa, Europe	Bird	Yes	Encephalitic
West Nile	Africa, Asia, Australia, the Caribbean, Europe, North America, South America	Bird	Yes	Encephalitic
Yaounde	Africa	Bird, rodent	Unknown	N/A

*One symptomatic report in a laboratory worker.

Cacipacore virus was originally isolated in 1977 from a bird in Brazil (Figueiredo et al., 2017). It is a zoonotic flavivirus that infects mosquitoes and has been detected in *Ae. aegypti* (Figueiredo et al., 2017). In 2011, CPCV was identified as the causative agent of a Brazilian farmworker who experienced acute febrile illness (Batista et al., 2011).

Koutango virus naturally infects rodents and mosquitoes. It was originally isolated from a rodent in Senegal, Africa in 1968. Transmission experiments have suggested that *Ae. aegypti* are a competent vector (de Araújo Lobo et al., 2014).

Murray Valley encephalitis virus has caused disease burdens in Australia and New Guinea (Mackenzie and Broom, 1995). *Culex* species mosquitoes are the primary vector with wading birds recognized as reservoir hosts. Humans infected with MVEV experience nonspecific symptoms such as fever and headache, but sometimes progresses into neurologic disease. Of the individuals who develop clinical disease 20% succumb to infection and many who develop symptoms do not fully recover, suffering from long term residual sequelae (Stich et al., 2003).

St. Louis encephalitis virus is named after an outbreak in St. Louis, Missouri in 1933 (Lumsden, 1958). Since then, SLEV has been found throughout the United States, Canada, and Mexico. *Culex* mosquitoes are the primary vector of SLEV with birds severing as reservoir hosts (Reisen, 2003). Human symptoms of SLE are generally nonspecific, though a subset can progress into neurologic disease. Of symptomatic cases, around 5-20% succumb to infection, with the majority of deaths observed in elderly patients (Reisen, 2003). In humans that recover, many can experience long term sequelae (Reisen, 2003).

West Nile virus was originally isolated from Uganda in 1937 and has since been detected across the globe (Smithburn et al., 1940). Until 1999, the majority of cases were in Africa and the Middle East. After introduction to the United States, WNV has been identified in hosts and vectors

in the 48 continental states, Canada, and Mexico. Wading birds are hosts for WNV with *Culex* species mosquitoes as primary vectors (Blitvich, 2008). Most humans infected with WNV (eight out of ten) do not develop symptoms and one in five people will experience nonspecific symptoms of fever, headache, and rashes (CDC, 2021a). More severe illness including encephalitis occurs in about one of every 150 people, with approximately one of every ten people who develop severe illness succumbing to the infection (CDC, 2021a).

Little is known about Yaounde virus, but it has been detected in *Culex* and *Aedes* mosquitoes in Africa (Braack et al., 2018). There have been no known cases of human disease, though rodents and birds can become infected (Richard et al., 2012).

Comparison of Japanese encephalitis virus and Usutu virus

Similarities between JEV and USUV warrant investigation of these two viruses to prevent their introduction to new continents. Both viruses are potentially serious threats for birds as well as mammals, including humans. These viruses share similar vectors, hosts, and potential for emergence (Table 1-3). When comparing identity at the nucleotide and amino acid level JEV exhibits 71% and 81% identity, respectively (Bakonyi et al., 2014).

Table 1-3. Comparison of Japanese encephalitis virus and Usutu virus.

	Japanese encephalitis virus	Usutu virus
Primary vector	*Culex* species mosquitoes	*Culex* species mosquitoes
Vertebrate hosts	Wading birds, pigs	Black birds, perching birds, owls
Emergence	Of dominant genotype (GIII to GI)	To novel locations
Geographic locations	Across south and southeast Asia, China, Indonesia, Northern Australia	Africa, Europe
Human disease	Encephalitic	Encephalitic

As the geographic location of JEV and USUV have expanded since original isolation, it is necessary to improve surveillance and determine the risk of introduction to other regions including North America. The establishment of WNV in the New World suggests that other flaviviruses primarily transmitted by *Culex* species mosquitoes such as JEV and USUV could potentially enter and become established in North America. To help control or stop the establishment of USUV, it will be important to identify the competency of North American mosquito species to USUV. Since it has previously been determined that North American mosquitoes are susceptible to JEV, it is important to assess the efficacy of current JE vaccines to protect humans (Huang et al., 2015). This includes the need to evaluate current JE platforms in a relevant animal.

Origin of Usutu virus

An emerging flavivirus warranting investigation is USUV. The USUV has been reported in multiple countries in Africa and has since been detected throughout Europe, causing high mortality rates in birds, particularly Eurasian blackbirds (Weissenböck et al., 2013; Williams et

al., 1964). Since USUV has been reported to co-circulate with WNV and is similar to WNV in its transmission, epidemiology, and clinical presentation, there is a risk of introduction to North America. North America has similar vectors, birds, and climates to Europe; and as JE serocomplex member WNV was able to become established in North America, it is possible that USUV could follow a similar trajectory.

Usutu virus clinical presentation

While historically the hundreds of reported USUV infections in humans have been associated with self-limiting febrile illness, there have been reports of USUV causing severe neurotrophic disease in humans (Cavrini et al., 2009; Grottola et al., 2017; Pecorari et al., 2009; Santini et al., 2015). The first human case was in the Central African Republic in 1981 (Nikolay et al., 2011). Human cases have since been reported in Italy in 2009 and more recently in Austria, France, and Germany (Allering et al., 2012; Bakonyi et al., 2017; Pecorari et al., 2009; Simonin et al., 2018). Some of the serious complications that have been reported in acute USUV infections are meningoencephalitis and facial paralysis. Also of concern is that human infections with USUV can be asymptomatic which can pose a problem for blood donations. This was demonstrated when USUV was detected in the blood of an asymptomatic donor (Bakonyi et al., 2017). As blood transfusions are utilized in immunosuppressed or critically ill patients, there is a risk of patients developing illness from USUV in endemic countries that do not screen blood donations for flaviviruses (Bakonyi et al., 2017).

Usutu virus transmission

The USUV transmission cycle often involves ornithophilic mosquitoes and birds. Originally isolated from a *Culex neavei* mosquito near the Usutu River in South Africa, other mosquito species reported in its transmission are primarily of the *Culex* genus (Williams et al. 1964). Isolations of USUV in field caught mosquitoes are listed in Table 1-4.

Table 1-4. Isolation of Usutu virus in field caught mosquitoes.

Species	Country	Year	References
Aedes albopictus	Israel	2015	(Mannasse et al., 2017)
	Italy	2009	(Calzolari et al., 2010)
Aedes japonicus	Austria	2018	(Camp et al., 2019)
Anopheles maculipennis	Italy	2010	(Mancini et al., 2017)
Coquilletidia aurites	Uganda	1962	(Williams et al., 1964)
Culex neavei	Senegal	2012	(Diallo et al., 2019)
	South Africa	1959	(Williams et al., 1964)
Culex perexiguus	Israel	2015	(Mannasse et al., 2017)
Culex pipiens	Austria	2018	(Camp et al., 2019)
	France	2015	(Eiden et al., 2018)
	Germany	2010	(Jost et al., 2011)
	Israel	2014	(Mannasse et al., 2017)
	Italy	2009	(Mancini et al., 2017)
	Serbia	2014	(Kemenesi et al., 2018)
	Spain	2006	(Busquets et al., 2008)
	Switzerland	2011	(Engler et al., 2013)
Culex univittatus	Uganda	2012	(Mossel et al., 2017)
Culiseta annulata	Italy	2011	(Mancini et al., 2017)
Ochlerotatus caspius	Italy	2010	(Mancini et al., 2017)
Ochlerotatus detritus	Italy	2011	(Mancini et al., 2017)

Experimental assessments of vector competence have also been performed and suggested transmission potential of several *Culex* species mosquitoes (Table 1-5). These studies have suggested *Aedes* species mosquitoes are less competent for transmission of USUV. However, further vector competence studies of North American mosquitoes are needed to determine if USUV could become established in the region.

Table 1-5. Vector competence experiments with Usutu virus.

Species	Blood meal titer $TCID_{50}$/mL	Infection	Dissemination	Transmission	Ref.
Aedes albopictus (Italy)	$0.66 \times 10^{7.5}$	0% (0/20)	N/A	N/A	(Puggioli et al., 2017)
	$0.66 \times 10^{7.5}$	0% (0/20)	N/A	N/A	
	$0.66 \times 10^{7.9}$	0% (0/19)	N/A	N/A	
Aedes japonicus (Virgina, USA)	6.4×10^{6}	7% (6/66)	0% (0/8)	0% (0/66)	(Bates et al., 2021)
Culex neavei (Senegal)	2.9×10^{7}	33.3% (1/3)	0% (0/1)	N/A	(Nikolay et al., 2012)
	2.9×10^{7}	22.2% (2/9)	0% (0/2)	N/A	
	2.9×10^{7}	0% (0/1)	N/A	N/A	
	2.5×10^{7}	90.9% (40/44)	40% (16/40)	81.3% (13/16)	
Culex pipiens (The Netherlands)	4×10^{7}	80%	N/A	69%	(Fros et al., 2015)
Culex pipiens (Virginia, USA)	1×10^{7}	9% (7/71)	0% (0/35)	0% (0/91)	(Bates et al., 2021)

Species	Blood meal titer $TCID_{50}$/mL	Infection	Dissemination	Transmission	Ref.
Culex pipiens (United Kingdom)	1.4×10^6	5% (1/20)	5% (1/20)	100% (1/1)	(Hernandez-Triana et al., 2018)
	1.4×10^6	0 % (0/18)	N/A	N/A	
Culex quinquefasciatus (BEI Resources NR-43025)	1×10^7	72% (56/76)	8% (6/76)	3% (2/76)	(Bates et al., 2021)
Culex restuans (Virginia, USA)	1×10^7	21% (4/19)	0% (0/8)	0% (0/19)	(Bates et al., 2021)

Birds are amplifying hosts of USUV, and it has been detected in various bird species in Africa and Europe (Nikolay, 2015; Nikolay et al., 2011). The largest burden seen in birds has been in Passeriformes, particularly black birds. Table 1-6 lists some of the birds and countries where USUV has been isolated. It is believed that migratory birds have facilitated the dispersal of USUV across both short and long distances (Engel et al., 2016). Both wild and domestic birds have been found to be susceptible to infection with USUV (Chvala et al., 2005; Chvala et al., 2004; Weissenböck et al., 2013). While USUV does not appear to cause severe disease symptoms in chickens, other bird species can succumb to organ failure, brain lesions, and the inability to fly (Chvala et al., 2005; Chvala et al., 2004). The USUV has also been detected in mammals (Bazanow et al., 2018; Cadar et al., 2014; Diagne et al., 2019; Escribano-Romero et al., 2015). The vector and host range of USUV could contribute to its geographic spread.

Table 1-6. Isolation of Usutu virus in birds.

Order	Common name	Countries	Reference
Coraciiformes	Common kingfisher	Germany	(Becker et al., 2012; Ziegler et al., 2015)
Passeriformes	Blackbird	Austria, Czech Republic, England, France, Germany, Italy, Morocco, the Netherlands, Switzerland	(Becker et al., 2012; Chvala et al., 2004; Figuerola et al., 2009; Folly et al., 2020; Hubálek et al., 2014; Lecollinet et al., 2016; Rijks et al., 2016; Steinmetz et al., 2011; Ziegler et al., 2015)
	Common starling	Germany	(Becker et al., 2012; Ziegler et al., 2015)
	Song thrush	Austria, Germany, Nigeria	(Chvala et al., 2004; Nikolay et al., 2011)
	Canary	Germany	(Becker et al., 2012; Ziegler et al., 2015)
	House sparrow	England, Germany, Switzerland	(Becker et al., 2012; Folly et al., 2020; Steinmetz et al., 2011)
	Blue tit	Austria, Germany, Switzerland	(Becker et al., 2012; Steinmetz et al., 2011)
	European greenfinch	Switzerland	(Steinmetz et al., 2011)

Order	Common name	Countries	Reference
	European robin	Austria, Switzerland	(Chvala et al., 2004; Steinmetz et al., 2011)
	Bullfinch	Belgium	(Garigliany et al., 2014)
	Nuthatch	Austria	(Chvala et al., 2004)
	Magpie	Italy	(Calzolari et al., 2010)
	Barn swallows	Switzerland	(Steinmetz et al., 2011)
Piciformes	Great spotted woodpecker	Belgium, Switzerland	(Garigliany et al., 2014; Steinmetz et al., 2011)
Strigiformes	Great grey owl	Germany, the Netherlands, Switzerland	(Becker et al., 2012; Rijks et al., 2016; Steinmetz et al., 2011; Ziegler et al., 2015)
	Snowy owl	Switzerland	(Steinmetz et al., 2011)
	Tengmaml's owl	Switzerland	(Steinmetz et al., 2011)
	Hawk owl	Switzerland	(Steinmetz et al., 2011)
	Pygmy owl	Switzerland	(Steinmetz et al., 2011)

Usutu virus geographic spread

Since its identification in South Africa, USUV has subsequently been found in many other countries in Africa and Europe (Fig. 1-4) (Cle et al., 2019; Williams et al., 1964). The USUV caused a major epidemic in northern Europe in 2016 and spread rapidly throughout western Europe in 2018 (Cle et al., 2019; Michel et al., 2018; Rijks et al., 2016). From its original isolation in a mosquito, USUV has since been detected in various vertebrate species in these countries including in birds, horses, and humans.

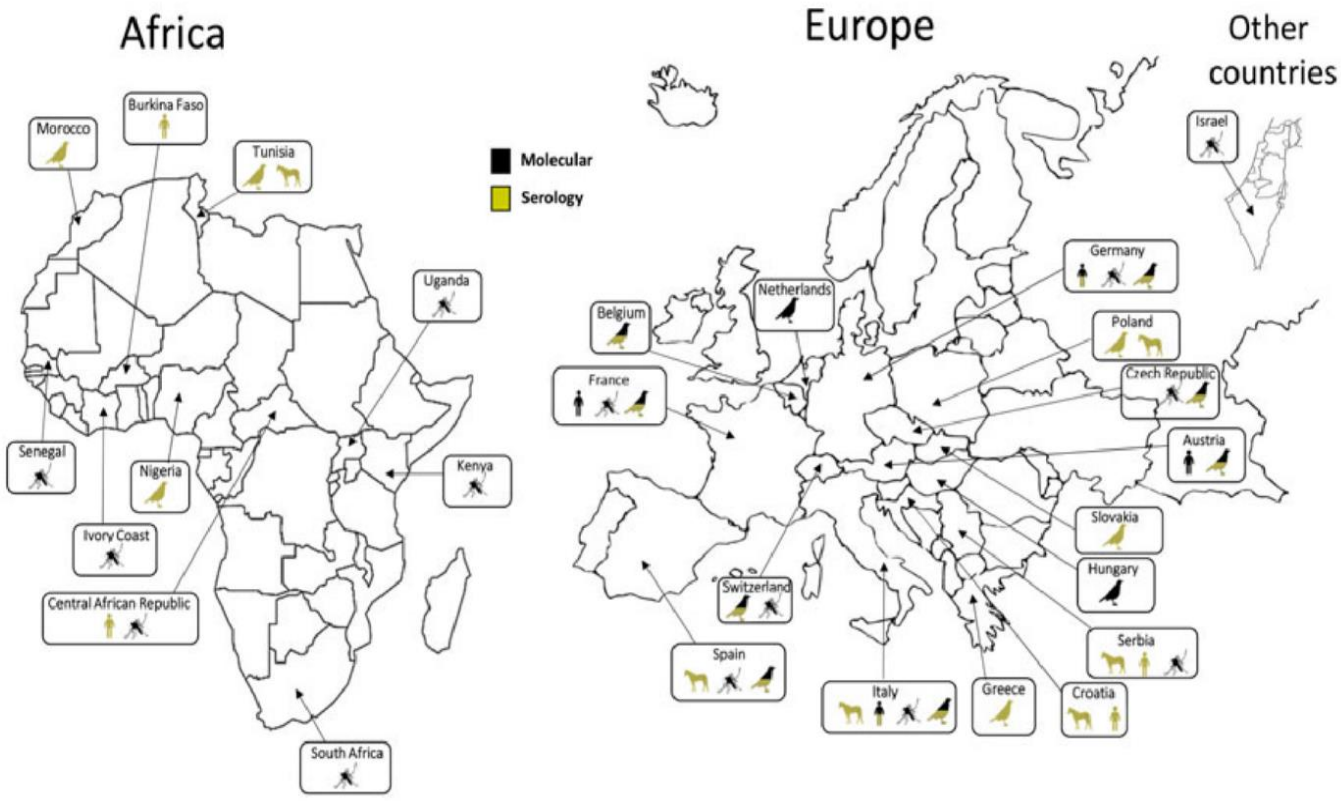

Figure 1-4. Usutu virus distribution with species and method of identification. Published under the Creative Commons (Cle et al., 2019).

As the geographic location of USUV has greatly expanded since being found in Africa, it is necessary to improve surveillance and determine the risk of introduction to other regions including North America. Table 1-7 lists countries where USUV has been isolated and the species it was detected in by the earliest year. With the occurrences of outbreaks in wildlife populations and increasing reports of human infections, USUV constitutes a public health concern to the New World. To begin to mitigate this risk, it will be important to identify the competency of North American mosquito species to USUV. The assessment of the susceptibility of key mosquito species to USUV needs to be determined in order to establish the role of North American mosquitoes in USUV transmission.

Table 1-7. Geographic spread of Usutu virus by year of earliest isolation.

Year	Country	Species	Reference
1959	South Africa	Mosquito	(Williams et al., 1964)
1962	Uganda	Mosquito	(Williams et al., 1964)
1969	Central African Republic	Human, mosquito	(Nikolay et al., 2011)
1972	Nigeria	Bird	(Cle et al., 2019)
1974	Senegal	Mosquito	(Nikolay et al., 2011)
1996	Italy	Bird, horse, human, mosquito	(Calzolari et al., 2013; Cavrini et al., 2009; Gaibani et al., 2012; Mancini et al., 2017; Weissenböck et al., 2013)
2001	Austria	Bird, human, mosquito	(Weissenböck et al., 2002)
2003	Hungary	Bird	(Bakonyi et al., 2007)
2004	Burkina Faso	Human	(Nikolay et al., 2011)
2006	Spain	Bird, horse, mosquito	(Ashraf et al., 2015; Busquets et al., 2008)

Year	Country	Species	Reference
2006	Switzerland	Bird, mosquito	(Engler et al., 2013; Steinmetz et al., 2011)
2007	Germany	Bird, human, mosquito	(Allering et al., 2012; Jost et al., 2011; Linke et al., 2007)
2007	Kenya	Mosquito	(Ochieng et al., 2013)
2008	Morocco	Bird	(Figuerola et al., 2009)
2008	Poland	Bird, horse	(Bazanow et al., 2018)
2011	Croatia	Horse, human	(Barbic et al., 2013; Santini et al., 2015)
2011	Czech Republic	Bird, mosquito	(Hubálek et al., 2014)
2012	Belgium	Bird	(Garigliany et al., 2014)
2012	Slovakia	Bird	(Csank et al., 2018)
2012	Tunisia	Horse	(Ben Hassine et al., 2014)
2014	Greece	Bird	(Chaintoutis et al., 2014)
2014	Israel	Mosquito	(Mannasse et al., 2017)
2014	Serbia	Horse, human, mosquito	(Cle et al., 2019; Kemenesi et al., 2018)
2015	France	Bird, human, mosquito	(Eiden et al., 2018; Lecollinet et al., 2016; Simonin et al., 2018)
2016	The Netherlands	Bird	(Rijks et al., 2016)

Usutu vaccines and treatments

Currently there are no licensed vaccines available against USUV and there is no treatment other than supportive care. Supportive care is mainly directed at symptoms and can include intravenous fluids and pain medication (Gill et al., 2019). Due to the lack of vaccine and treatment options, prevention is the primary way to prevent disease for example, avoiding being bitten by potential vectors.

Animal models for Usutu virus research

With the emergence of USUV to new geographic locations, several mouse models have been assessed to investigate USUV. Wild type mice have shown limited susceptibly to USUV infection unless the mice are young, under a week old (Weissenböck et al., 2004). Clinical signs of USUV observed in the young mice have included disorientation and paralysis. Immunodeficient mice, and mice such as AG129 deficient in interferon receptors, challenged with USUV experienced severe clinical symptoms and death (Kuchinsky et al., 2020; Segura Guerrero et al., 2018).

Avian species have also been assessed for understanding USUV pathogenesis and transmission within bird hosts. This included investigation of domestic Austrian geese and domestic German chickens. Both species were demonstrated to be susceptible to USUV *in vivo,* with detection in organ samples and pharyngeal swabs, though neither species developed viremia (Chvala et al., 2005; Chvala et al., 2004). Domestic canaries from Belgium were also evaluated for their susceptibility to infection with USUV, and USUV was detected in the blood and feces (Benzarti et al., 2020). Juvenile domestic chickens from Virginia were determined to be susceptible to USUV, developing viremia and shedding USUV in oral and cloacal secretions

(Kuchinsky et al., 2021). These avian platforms could provide support to evaluate USUV in a host species and assist in determining the threat of USUV to birds in novel geographic locations.

The development of *in vivo* models for USUV is relatively new, as human infections have historically been asymptomatic. However, with the increase in human infections and impact on the bird population more research is being conducted on USUV. With the potential of USUV to continue to emerge, more studies of USUV animal models could be conducted in the future.

Current challenges and gaps in knowledge

The introduction, spread, and establishment of USUV into new geographic locations has made it a public health concern. Current risks and challenges include managing potential large outbreaks where USUV is already recognized and preventing USUV from becoming established in new areas. This will require the development and implementation of USUV surveillance and control measures. The evaluation of vector and host competency can help determine regions where USUV would be a threat and aid in creation of prevention and control strategies.

Origin of Japanese encephalitis virus

The other JE serocomplex member of focus in this dissertation is JEV, which is a mosquito-borne virus that causes JE in humans. Historical descriptions of humans exhibiting clinical disease symptoms similar to JE were described as early as 1871, with JEV first isolated from brain tissue of a fatal human encephalitic case in Tokyo, Japan in 1935 (Solomon et al., 2003). The virus was later isolated from *Culex tritaeniorhynchus* mosquitoes in 1938, and has since continued to be a threat to public health (Hammon and Tigertt, 1949). There are over three billion people at risk of

infection with JEV in the regions where it is currently endemic, and over 68,000 cases of JE estimated annually (CDC, 2019).

Presently, treatment of JE is mainly supportive, making efforts toward prevention and control primarily geared towards vaccination and the reduction of exposure to mosquitoes. As the leading cause of vaccine-preventable encephalitis in Asia and the western Pacific, improvements are needed to assess JE vaccine efficacy and coverage (CDC, 2019).

Japanese encephalitis clinical presentation

Human clinical presentation

Human infections with JE can range from asymptomatic to symptomatic. Nonspecific febrile illness is generally the first symptom which can be followed by headaches, vomiting, and disorientation (Solomon et al., 2000; Weaver and Barrett, 2004). Severe clinical disease occurs in approximately 1% of people infected with JE and can progress to encephalitis, seizures, and neurologic deficits (WHO, 2019). Around 20-30% of clinical cases of JE result in death, while survivors can experience severe long term side effects such as paralysis and cognitive deficits (Solomon et al., 2000). In endemic regions children are typically the most susceptible to displaying symptomatic JE infection (Borah et al., 2011). This could be due to the absence of previous immunity coupled with exposure to competent vectors while playing outside.

Swine clinical presentation

Clinical signs of JE in pigs typically vary by age with the greatest impact observed in young swine. Infected piglets can suffer from various neurologic symptoms and ultimately death (Yamada et al., 2004). The greatest impact of JE in adult swine is generally observed through reproductive losses such as decreased boar sperm quality and abortion in sows (Burns, 1950; Imoto

et al., 2010; van den Hurk et al., 2009). Experimental challenge of swine with JEV demonstrated that viremia can occur as early as one day post infection and can last around three days with fevers also observed (Park et al., 2018; Ricklin et al., 2016a). The tissue tropism of JEV has also been assessed in experimentally infected swine and found JEV within the lymphoid and central nervous system tissues (Park et al., 2018; Ricklin et al, 2016). The clinical symptoms and pathology of JEV infection in pigs suggests their relevance to study JEV *in vivo*.

Japanese encephalitis virus transmission

The transmission of JEV is maintained with *Culex* species mosquitoes as the vector and avian and swine species as amplification hosts (van den Hurk et al., 2009). *Culex tritaeniorhynchus* have been considered the most significant vector for JEV in Southeast Asia, though JEV has been isolated in many other species of field caught mosquitoes (Table 1-8). Ornithophilic, bird feeding, mosquitoes act as bridge vectors for JEV in rural locations with open water sources serving as breeding grounds. Many *Culex* spp. mosquitoes are ornithophilic but will also feed on both livestock and humans.

Table 1-8. Species and location of the isolation of Japanese encephalitis virus in field caught mosquitoes with earliest isolation year.

Species	Location	Year	References
Aedes albopictus	Malaysia	1992	(Vythilingam et al., 1995)
	Taiwan	1995	(Su et al., 2014; Weng et al., 1999)
Aedes vexans	Taiwan	1995	(Weng et al., 1999)
Aedes vigilax	Australia	1997	(Hanna et al., 1999)
	China	2007	(Feng et al., 2012)

Species	Location	Year	References
Armigeres subalbatus	Taiwan	1995	(Weng et al., 1999)
Culex annulirostris	Australia	1995	(Ritchie et al., 1997)
Culex bitaeniorhynchus	Malaysia	1992	(Vythilingam et al., 1995)
	South Korea	2008	(Takhampunya et al., 2011)
Culex fuscocephala	India	1985	(Mourya et al., 1989)
	Indonesia	1978	(Olson et al., 1985)
	Malaysia	1992	(Vythilingam et al., 1997)
	Sri Lanka	1987	(Peiris et al., 1992)
	Taiwan	1974	(Hsu et al., 1978)
	Thailand	1970	(Gould et al., 1974)
Culex gelidus	Australia	2000	(van den Hurk et al., 2001)
	India	1985	(Mourya et al., 1989)
	Indonesia	1972	(Dirk Van Peenen et al., 1975)
	Malaysia	1954	(Gould et al., 1962)
	Sri Lanka	1987	(Peiris et al., 1992)
	Thailand	1962	(Simasathien et al., 1972)
	Vietnam	1972	(Thi-Kim-Thoa et al., 1974)
Culex pipiens	China	2015	(Shi et al., 2019)
	Italy	2010	(Ravanini et al., 2012)
	Japan	1952	(Buescher et al., 1959b)
	South Korea	2008	(Kim et al., 2015)
Culex pseudovishnui	India	1977	(George et al., 1987)
Culex quinquefasciatus	India	1985	(Mourya et al., 1989)
	Taiwan	1995	(Weng et al., 1999)
	Thailand	2003	(Nitatpattana et al., 2005)
	Vietnam	1972	(Thi-Kim-Thoa et al., 1974)

Species	Location	Year	References
Culex sitiens	Australia	1998	(Johansen et al., 2001)
	Malaysia	1992	(Vythilingam et al., 1995)
	New Guinea	1997	(Johansen et al., 2000)
	Taiwan	1995	(Weng et al., 1999)
Culex tritaeniorhynchus	Cambodia	2014	(Duong et al., 2017)
	China	2005	(Sun et al., 2009)
	India	1962	(Carey et al., 1968)
	Indonesia	1972	(Olson et al., 1985)
	Japan	1964	(Yamamoto et al., 1970)
	Malaysia	1968	(Simpson et al., 1974)
	Taiwan	1969	(Okuno et al., 1971)
	Thailand	1962	(Simasathien et al., 1972)
	Singapore	2011	(Yap et al., 2020)
	Sri Lanka	1987	(Peiris et al., 1992)
	South Korea	2008	(Takhampunya et al., 2011)
	Vietnam	1972	(Thi-Kim-Thoa et al., 1974)
Culex vishnui	India	1975	(Chakravarty et al., 1975)
	Indonesia	1978	(Olson et al., 1985)
	Malaysia	1992	(Vythilingam et al., 1995)

Experimental studies have been conducted to evaluate vector competence for JEV, including studies in North American mosquitoes (Table 1-9). While JEV has not been detected in the New World, work in our lab demonstrated that North American *Cx. quinquefasciatus* are susceptible to JEV infection and could be able to contribute to its establishment in the region (Huang et al., 2015). This highlights the importance of continuing to improve flavivirus prevention and control measures as they could become established in new geographic locations.

Table 1-9. Vector competence of North American mosquitoes for Japanese encephalitis virus.

Species	Strain	Blood meal	Transmission detected	Number of days virus detected	Ref.
Aedes dorsalis	Nakayama (GIII)	Virus rabbit blood suspension	1	16	(Reeves and Hammon, 1946)
Aedes nigromaculis			4	16	
Culex molestus			3	20	
Culex pipens			2	21	
Culex quinquefasciatus			4	26	
Culex tarsalis			2	25	
Culiseta incidens			3	20	
Culiseta inornata			3	20	

Species	Strain	Blood meal titer $logTCID_{50}/mL$	Infection	Dissemination	Transmission	Ref.
Culex quinquefasciatus (Valdosta, GA)	Taira (GIII)	4.8	7 DPI 100% (12/12) 14 DPI 84.6% (22/26)	7 DPI 0% (0/8) 14 DPI 50% (7/14)	9.1% (2/22)	(Huang et al., 2015)
Culex quinquefasciatus (Valdosta, GA)	KE-93-83 (GI-a)	7.99±0.41	7 DPI 43.9% (18/41) 14 DPI 35.2% (19/54)	7 DPI 23.1% (3/13) 14 DPI 16.7% (2/12)	5.3% (1/19)	(Huang et al., 2016)
Culex quinquefasciatus (Valdosta, GA)	JE-91 (GI-b)	8.13±0.72	7 DPI 57.6% (19/33) 14 DPI 55.6% (25/45)	7 DPI 30.0% (3/10) 14 DPI 28.6% (4/14)	8.0% (2/25)	(Huang et al., 2016)
Culex quinquefasciatus (Valdosta, GA)	Tairia (GIII)	8.36±0.53	7 DPI 95.1% (39/41) 14 DPI 66.7% (44/66)	7 DPI 8.3% (2/24) 14 DPI 32.1% (9/28)	6.8 % (3/44)	(Huang et al., 2016)

Pigs are the primary mammal known to serve as an amplification host of JEV. Pigs develop high viremia and are able to infect feeding mosquitoes (Solomon, 2006). Pigs are major contributors to the JEV transmission cycle in East and Southeast Asia due to their quick viral replication and the intensive pig farming in the region (Erlanger et al., 2009). Many of these farms have large pig herds and the proximity between humans and livestock can create an increased risk of JE (Erlanger et al., 2009). Both domestic and wild pigs are susceptible to infection, which could aid in the transmission of JEV in new locations (Park et al., 2018; Sota et al., 1991).

Among avian species, wild wading birds, (in particular egrets and herons) are highly susceptible to JEV (Le Flohic et al., 2013). Wading birds are considered a major reservoir and amplification host of JEV as they can develop high-titer viremia and facilitate transmission to mosquitoes (Buescher et al., 1959a; Burke et al., 1985). The migratory nature and wide distribution of these avian species can contribute to the spread of JEV across countries (Nga et al., 2004).

The presence of susceptible vectors and hosts across the world necessitate continued assessment of JE surveillance and prevention measures to prevent its introduction. It has previously been determined that North American mosquitoes, swine, and birds are susceptible to JEV infection, increasing the risk of JEV establishment if it were to be introduced (Huang et al., 2015; Nemeth et al., 2012; Park et al., 2018). The introduction of the JEV to North America could be devastating as the disease would have negative impacts on human and animal health.

Swine oronasal shedding

Recent laboratory discoveries indicate the transmission of JEV can occur without competent arthropod vectors. Studies have demonstrated the ability of swine to shed JEV in oronasal secretions that spread through direct contact when co-housing infected and

immunologically naïve pigs (Lyons et al., 2018; Ricklin et al., 2016a). Domestic pigs were found to be susceptible to JEV through direct contact with minimum infectious dose for establishment of infection as low as 10 tissue culture infectious dose 50 ($TCID_{50}$) (Ricklin et al., 2016a). This suggests that pigs have the ability to maintain the transmission of JEV without the presence of competent vectors, which could contribute to outbreaks of JE related abortions and stillbirths in swine even when mosquito populations are low (Ricklin et al., 2016a; Takhampunya et al., 2011).

The shedding of JEV in swine oral fluids could allow for an alternative method to determine infection in pigs. The collection of oral fluid using rope-based methods has benefits over traditional diagnostic samples such as blood and swabs as it is less labor intensive, less intrusive to the animal, cheaper, and allows for the sampling of multiple animals. With the potential for swine to shed JEV in oronasal secretions, these samples could be of interest in laboratory experiments and for use in surveillance and evaluation of pig herds and could support the use of swine vaccinations in endemic locations. The oronasal shedding of JEV in swine also suggests benefits of swine to study disease pathogenesis to assess vaccines and therapeutics for reduction of oronasal secretion viral load and could be utilized for future assessment of JE swine vaccines.

Japanese encephalitis virus geographic spread and genotypes

Epidemics of JE have been described in Japan since the 1870s and since then, JEV has spread throughout most of Asia and the Pacific region (Figure 1-5). While JEV has only one serotype, there are five distinguishable genotypes: genotype I (GI), genotype II (GII), genotype III (GIII), genotype IV (GIV), and genotype V (GV). The variation in the amino acids of the E protein

between JEV strains in GI, GII, GIII, and GIV is less than 6%, and with the addition of the Muar strain (GV), the difference between strains is 12% (Tsarev et al., 2000).

Until the 1990s, GIII was the most prevalent genotype of JEV, but in the last few decades, it has been displaced with GI strains emerging in several regions (Do et al., 2015; Huang et al., 2016b). The GI strains were originally only endemic in Southeast Asia but have since spread across most of the continent (Gao et al., 2019; Pan et al., 2011). Two clades make up GI, GI-a and GI-b. The GI-a was continuously prevalent in the tropical regions of Asia, while GI-b has replaced GIII as the dominant genotype in Asia (Schuh et al., 2014).

The GII strains are typically found between northern Australia and southern Thailand and have caused several human fatalities (Schuh et al., 2010). The earliest JEV isolate that was obtained from Tokyo, Japan was a GIII isolate, and GIII strains have since been utilized in the creation and development of JE vaccines. Strains of GIII have been associated with many epidemics of viral encephalitis in humans. As of 2017, GIV has only been isolated from Indonesian mosquitoes between 1980 and 1981, and has not been linked to cases of viral encephalitis in humans (Schuh et al., 2013). The GIV has the most limited distribution of the JEV genotypes. Currently, only a few isolates of GV have been reported (Gao et al., 2019).

The five JEV genotypes differ by their geographic locations, as well as climatic conditions. The GI-a and GII have been primarily found in the tropics, while GI-b and GIII have been located in regions that are more temperate. Currently, GIV has only been found in tropical regions and GV has been discovered in areas with both temperate and tropical climates (Schuh et al., 2013). As the dominant genotype of JEV can change and vary by geographic location, it is important to continue to evaluate vaccine and therapeutic candidates. Current JE vaccines derived from GIII

strains might not provide complete protection against the emerging GI strains. The ideal vaccine candidate would be able to universally protect against multiple JEV genotypes.

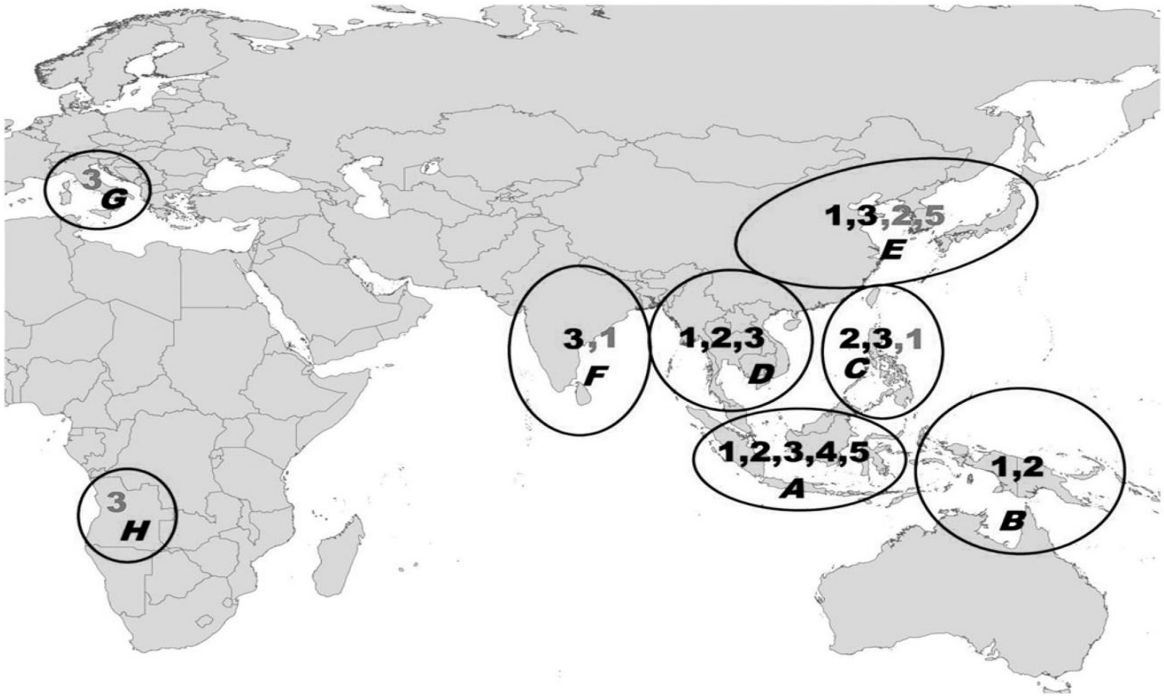

Figure 1-5. Geographic distribution of JEV genotypes 1-5. A-F: traditional epidemic areas of JEV within Asia and Oceania with JE emergence before 2000. G and H: Italy and Angola; areas that were previously considered non-epidemic with emergence of JEV after 2000. The numbers correspond with the JEV genotypes endemic in the region. Black numbers are JEV genotypes prevalent before 2000 and gray after. Published under the Creative Commons (Gao et al. 2019).

Japanese encephalitis vaccines and treatments

Due to the lack of effective treatments, vaccination is the most effective approach for disease control and prevention of JE. Existing JE vaccine platforms are derived from GIII strains and are inactivated or live-attenuated (Table 1-10). These vaccines have helped to decrease JE disease burdens.

Table 1-10. Summary of Japanese encephalitis vaccines. All are derived from genotype III strains.

Vaccine type	Substrate	Virus strain	Trade name	Manufacturer	Availability
Inactivated	Mouse brain	Nakayama	JE-Vax	Vabiotech (Vietnam)	Vietnam
	Vero cells	Beijing-1	BK-VJE	Biken (Japan)	Japan
	Vero cells	Beijing-3	P-3	Liaoning Chenga Bitech (China)	China
	Vero cells	SA14-14-2	IXIARO	Valneva (Austria)	European Union and United States
	Vero cells	Kolar 821564XY	JenVac	Bharat Biotech (India)	India
Live-attenuated	Primary hamster kidney	SA-14-14-2	CDJEVAX	Wuhan Institute of Biological Products (China)	Asia

Vaccine type	Substrate	Virus strain	Trade name	Manufacturer	Availability
Live-attenuated chimeric	Vero cells	JE SA-14-14-2 and yellow fever 17D	IMOJEV	Sanofi Pasteur (France)	Australia, Malaysia, Taiwan, Thailand

The development of first generation JE vaccines began shortly after the causative agent was discovered, with an inactivated mouse brain derived vaccine in the 1940s (McArthur and Holbrook, 2011). Specifically, JE-VAX®, an inactivated mouse brain vaccine, was licensed in Japan in 1954 (Hoke et al., 1988; Igarashi, 2002). The mouse brain derived vaccines are obtained from intracranially inoculating young mice with the chosen infectious strain and then harvesting, purifying, and formalin inactivating the virus. These vaccines require three doses with subsequent boosters, and adverse vaccine reactions have been reported.

The SA14-14-2 vaccine virus strain was created through serial passages of the wild type SA14 strain that was isolated from *Cx. pipiens* larvae in China in 1954 (Yu, 2013). The steps of attenuation are outlined in Table 1-11. The passages of the SA-14 strain in primary hamster kidney cells led to the isolation of the SA14-5-3 strain (Barrett, 2001). The SA14-5-3 strain was evaluated for use in humans, though it was not immunogenic after one dose; this led to the SA14-5-3 strain to be passaged further to derive the SA14-14-2 strain (Barrett, 2001).

Table 1-11. Passage history of the SA14-14-2 vaccine. Modified from (Monath, 2002).

SA14 virus isolated from *Culex pipiens* larvae from 11 passages in mouse brain
100 passages in primary hamster kidney cells
Five passages in chick embryo cells
One passage in mice, spleen harvested for plaque purification in chick embryo cells
Three plaque purifications in primary chick embryo cells
One passage in mice, skin harvested for plaque purification in chick embryo cells
Six oral passages in hamsters, spleens harvested for two plaque purifications in primary hamster kidney cells
Five passages in suckling mice, skin harvested for two plaque purifications in primary hamster kidney cells

There are inactivated and live-attenuated JE vaccines that have been established using the SA-14-14-2 strain. Inactivated platforms include the Vero cell derived vaccine IC51 that has been licensed under the trade name IXIARO®. The inactivated IXIARO® vaccine is the only JE vaccine licensed and available for use in the United States, with two doses of the vaccine required administered 28 days apart (Yun and Lee, 2014). The live-attenuated SA14-14-2 vaccine was first licensed in China in 1988 (Yu, 2013). This vaccine has been used for over 30 years and constitutes more than half of the global production of all JE vaccines, with more than 50 million doses produced each year (WHO, 2005). Only one dose of SA14-14-2 is required, which is an advantage over multiple dosage vaccine platforms when vaccinating remote endemic areas. The live-attenuated SA14-14-2 vaccine has been determined to have effectiveness of 80% for a single dose and 97.5% for a two-dose regimen when given 12 months apart (Hennessy et al., 1996).

A live-attenuated vaccine using yellow fever 17D (YFV-17D) vaccine as a vector called ChimeriVax-JE has also been developed. The ChimeriVax-JE vaccine has been demonstrated to protect monkeys against lethal JEV challenge (Monath et al., 2003; Monath et al., 2000). This

vaccine uses the YFV-17D as a genetic backbone with substitutions of the prM and E genes of SA14-14-2. ChimeriVax-JE has demonstrated safety and immunogenicity, requiring a single dose (Monath et al., 2003; Monath et al., 2000).

Despite the implementation of JE vaccines, 81% of infections occur in countries with vaccination programs (Campbell et al., 2011; Erlanger et al., 2009). This could be due to the lack of vaccine coverage, inability to maintain a JE vaccine supply, and poor surveillance (Vannice et al., 2021). The three of these are interlinked, and improvements should help decrease JE disease. For surveillance this would include assessing JE vaccine programs to determine long term protection and geographic spread (Vannice et al., 2021). Currently, there are no licensed antiviral therapies for JE. Treatment is mainly supportive and directed at managing symptoms. However, investigations of mAbs could support the development of JEV treatment options in the future.

The impact of the shift of Japanese encephalitis virus genotypes on vaccines

It is unknown if the current JE vaccines will be able to reduce disease burdens from emerging GI strains. Vaccine trials have often been limited to testing efficacy against GIII strains, as it has historically been the dominant JEV genotype. Since the licensed JE vaccines are derived from GIII strains, it is critical to continue to evaluate and develop vaccines with the ability to protect against multiple genotypes. This is supported with the increase of GI isolations in vertebrate hosts and mosquitoes (Schuh et al., 2014). Additionally, North American *Cx. quinquefasciatus* could be competent vectors if JEV GI strains were to enter the continent (Huang et al., 2016b). Evaluation of the GIII derived SA14-14-2 vaccine demonstrated that it exhibited low protective efficacy against GV challenge, with a 50% survival rate in mice (Cao et al., 2016). Protection of the SA14-14-2 vaccine has also been assessed against GI challenge and was partially protective, between 60-80% in mice (Wei et al., 2019). While these studies suggest limitations of

the current GIII derived vaccines, future studies should assess vaccine efficacy against GI strains in a natural host of JEV.

Monoclonal antibodies against Japanese encephalitis virus

Similar to other flaviviruses, the humoral immune response and neutralizing antibodies are needed to limit infection with JEV. With the lack of treatment options against JEV, mAbs have been developed to evaluate therapeutic candidates. The impact which the shift in JEV genotypes has on the efficacy of current vaccines has also begun to be assessed through evaluations of mAbs. Details of mAbs against JEV are described in Table 1-12. The described anti-JEV mAbs are against the E protein, which is the principal antigen that elicits neutralizing antibodies against infection (Pierson et al., 2008).

The JEV mAbs 7E5 and 3-3H8 were found to be reactive with GI and GIII strains *in vitro* (Shimoda et al., 2013). There has also been the generation of cross neutralizing humanized mAbs that were derived from immunized chimpanzees that were able to provide prophylactic protection *in vivo* in mice against heterologous strains of the same genotype (Goncalvez et al., 2008). The anti-JEV mAb 17BD3-2 was generated from mice immunized with the IXIARO vaccine and EDIII and demonstrated to be neutralizing *in vitro* against JEV GI, GII, and GIII (Calvert et al., 2019). An EDIII linear epitope, 2B4, has been described as neutralizing *in vitro* as well as partially protective against challenge in mice (Deng et al., 2014). An additional anti-JEV mAb that has been evaluated is the T16 mAb that is a serocomplex cross-reactive antibody, neutralizing JEV and WNV (Fan et al., 2015).

Recently a panel of mAbs against JEV was generated after immunizing mice with the GIII vaccine strain SA-14-14-2 (Fernandez et al., 2018). These mAbs were evaluated for neutralization *in vitro* against genotypes I, II, III, and IV by Fernandez et al. The mAbs were additionally

evaluated to determine if they were able to protect against JE infection from GI and GIII strains in a mouse model. This was assessed through a prophylactic dose mAbs given prior to challenge with virulent virus and several of the mAbs were determined to provide protection against one GIII and two GI strains. Despite the aforementioned strides, further evaluation is needed to provide insight into the development and efficacy assessment of current JE vaccines against emerging JEV genotypes.

Table 1-12. Monoclonal antibodies against Japanese encephalitis virus

mAb	Source	Immunization	Residues	Domain	Reference
2F2	CXS mice	SA14	52, 55, 123, 124, 128, 134, 205, 275, 277	EDI, EDII, EDIII	(Qiu et al., 2018)
2H4			52, 55, 123, 124, 128, 134, 205, 275		
7E5	BALB/c mice	JEV/sw/Chiba/88/2002	52, 276	EDI-EDII hinge	(Shimoda et al., 2013)
3-3H8			52	EDI-EDII hinge	
A3	Chimpanzee	JE-VAX and SA14-14-2	179	EDI lateral ridge	(Goncalvez et al., 2008)
B2			126	EDI-EDII hinge	
E3			302	EDIII lateral ridge	

mAb	Source	Immunization	Residues	Domain	Reference
2B4	BALB/c mice	JEV E, EDIII	394-397	EDIII	(Deng et al., 2014)
T16	BALB/c mice	Nakayama formalin inactivated vaccine	329, 331, 389	EDIII lateral ridge	(Fan et al., 2015)
17BD3-2	BALB/c mice	JEV IXIARO vaccine, EDIII	309, 312, 333	EDIII lateral ridge	(Calvert et al., 2019)
17BD3-6					
JEV-106	Irf3−/− mice	SA-14-14-2	333	EDIII lateral ridge	(Fernandez et al., 2018)
JEV-128			308, 310, 332, 333, 379, 396	EDIII lateral ridge	
JEV-131			308, 310, 332-334, 345, 379, 382, 396	EDIII lateral ridge	
JEV-143			308, 310, 332, 333	EDIII lateral ridge	

Animal research models for Japanese encephalitis

Traditionally murine models have primarily been used for *in vivo* JE models. Though there are multiple factors including genetic background and route of inoculation that can impact outcomes, young mice are particularly prone to JE infection (Grossberg and Scherer, 1966; Kimura et al., 2010). Suckling mice are particularly susceptible to JEV, with high fatality rates when

challenged intracerebrally or peritoneally (Kimura et al., 2010). The route of inoculation can influence JE disease signs, with intracerebral infection of mice not causing inflammation, though this could be due to the rapid onset of death in these animals within several days (Hase et al., 1990). Intraperitoneal inoculation of mice has been demonstrated to not cause death in all animals, but inflammation occurs. The intraperitoneal challenge route is used more often than subcutaneous routes because mice inoculated intraperitoneally are more susceptible to JEV infection (Kimura et al., 2010). Though not utilized as often, hamsters and rats are other small animal JE *in vivo* models, while rabbits and guinea pigs have been shown to experience asymptomatic infection (Burke, 2001; Nalca et al., 2003).

Rhesus and macaque monkeys are examples of nonhuman primates that have been used to study JEV. The route of inoculation impacts the outcome of infection in monkeys, with intracerebral JEV challenge usually leading to lethal encephalitis (Monath et al., 1999). Intradermal inoculation of JEV in rhesus monkeys caused viremia but not fatal encephalitis, while intranasal inoculation of JEV caused viremia, encephalitis, and death in macaques (Lee et al., 1967; Morris et al., 1955). The homology between humans and nonhuman primates suggests the usefulness of the macaque model for the evaluation of flavivirus vaccines and therapies, though investigation of an animal model that could resemble human infection through similar challenge routes would be valuable.

Potential of swine to study Japanese encephalitis *in vivo*

The ideal animal model for studying a human disease would accurately reproduce aspects of the disease being investigated. Animal models are critical for vaccine development, providing valuable information such as dosage, toxicity, and efficacy. For vaccine preclinical vaccine

development, it is especially important to select the most appropriate animal model available that can closely resemble the target species.

In recent years, swine have become increasingly important in assessing vaccines and therapeutics because of their similarity to humans in terms of their anatomy and physiology (Heinritz et al., 2013; Kararli, 1995; Perleberg et al., 2018). This includes size, dietary habits, and hairless phenotype in some species. The large size of pigs has led to miniature or micro pigs becoming desirable for research use. The advantages of miniature swine breeds include the ability to model human disease symptoms and slow growth. The size of a miniature pig can make them more effective than a small animal model because they can be repeatedly sampled, with larger volumes taken at each time point; while they can also be less expensive compared to their full-size counterparts, requiring less space and food.

There are several different miniature swine breeds currently used in the United States that include Hanford, Sinclair, and Yucatan breeds. Of particular interest are the Yucatan miniature pigs that are naturally occurring miniature swine native to the Yucatan Peninsula known for their sparse coats and short snouts (Panepinto, Phillips, Wheeler, & Will, 1978). They are known for their docile temperament and their lack of hair makes them ideal for laboratory settings and procedures such as injections and blood draws (Panepinto & Phillips, 1986).

Given they are amplification hosts of JEV, swine could be utilized to study JE, which would have advantages when testing JE vaccine efficacy. Pigs also have the potential to develop pathological outcomes of JE as observed in humans, experiencing appropriate biological markers to infection including clinical signs and tissue tropism (Park et al., 2018; Ricklin et al., 2016b). Miniature swine could be quickly employed if a JE outbreak were ever to occur in the United States or other novel location.

Current challenges and gaps in knowledge

Although vaccines against JEV are available, there are still challenges controlling and preventing JE. Gaps in knowledge include the effectiveness of JE vaccines, currently all GIII derived, against multiple genotypes. It is unknown if JE vaccines will be effective if new genotypes emerge. There are no specific treatment options for JE, which will be important to address in the future to help patients with poor clinical outcomes. To effectively evaluate vaccines and therapeutics investigation of an immunocompetent animal model is needed. Miniature swine could address difficulties observed in other animal models by demonstrating similar disease outcomes as human infection through challenge routes applicable to natural infection. There is also a need to improve JE surveillance; this will assist with JE vaccination challenges, determining the effectiveness of vaccine programs and monitoring the geographic spread.

Justification of the research

The growing human population and establishment of flaviviruses in previously non-endemic areas, suggests a global health problem involving one or more of these viruses is imminent. Therefore, it is imperative to evaluate and improve surveillance, control, and prevention measures. Since JE serocomplex members are transmitted by mosquito vectors, research involving the susceptibility of mosquitoes in non-endemic regions is essential. In addition, the ever-increasing threats of new flavivirus infections and neurologic sequalae make the development of effective vaccines important to public health.

Specifically, flaviviruses of the JE serological group including JEV and USUV are of public health importance due to their propensity to emerge and cause disease in animals and humans. The need to evaluate the competency of North American mosquitoes to emerging

flavivirus USUV was highlighted with the introduction of WNV to North America in 1999 because it demonstrated that flaviviruses can become established in novel geographic locations when appropriate combinations of vectors and vertebrate hosts are present. Similarities between USUV and WNV including host and vector species emphasize the need to assess its emergence potential. Evaluations of the vector competency of mosquitoes present in the United States can help control the spread of disease and be used to deter them from becoming endemic.

While knowledge of potential vector populations will aid in flavivirus control, it is also critical to assess flavivirus vaccines. Currently, flaviviruses cause large disease burdens and outbreaks each year, necessitating improved personal preventative measures. Specifically, JEV has been estimated to cause 68,000 human clinical cases and approximately 13,600 to 20,400 deaths each year (WHO, 2019). The potential severity of JE and USUV clinical symptoms and risk of disease outbreak necessitate the continued assessment of vaccines to evaluate potential broad-spectrum protection.

Current licensed vaccines for JE are derived from GIII strains despite the emergence of GI as the dominant genotype throughout Asia. With the changing dynamics of dominant JEV genotypes and potential emergence to novel locations, it is also necessary to establish an appropriate animal model for flaviviruses research and vaccine development purposes. This can be achieved through the determination of an animal model that can verify the protective effect of flavivirus vaccines and demonstrate that the protection correlates with neutralizing antibodies. The testing needed to demonstrate safety and efficacy of vaccines requires a higher order animal species after *in vitro* and murine model work. Studies have demonstrated that humans and pigs infected with JEV share similar disease symptoms, including the development of neurotropic disease (Park et al., 2018; Ricklin et al., 2016b; Yamada et al., 2004). This implies that swine

could represent a biologically relevant platform for the preclinical development of therapeutic and vaccine candidates, as well as the continued evaluation of vaccine efficacy. The development of pathological outcomes in pigs could provide the basis necessary for the demonstration of protective efficacy *in vivo* and serve as a relevant alternative to other animal models.

The long-term goal of the project is to improve flavivirus prevention and control through identification of susceptible vectors and assessment of antibody efficacy which could aid in personal preventative measures, such as vaccination platforms. The major objective of the research outlined in this dissertation is to evaluate prevention and control measures against two JE serocomplex members to mitigate against potential introduction into the North America. Specifically, these goals will be addressed through determining if North American mosquitoes are capable of transmitting emerging flavivirus USUV and assessing the protective efficacy of vaccination derived anti-JEV mAbs in miniature swine. The central hypothesis for the dissertation is that **the identification of competent mosquito species and the evaluation of neutralizing antibodies in a natural host are both fundamentals needed to improve flavivirus prevention, control, and mitigation measures.**

The following specific aims will be used to test this hypothesis:

Specific aim 1: Determine the susceptibility and competency of North American mosquitoes to USUV.

- Evaluate infection and dissemination in mosquito species found in North America.
- Find the presence or absence of viral RNA in saliva of infected mosquito species to ascertain transmission competency.

Specific aim 2: Determine the *in vivo* protection of an anti-EDIII mAb in miniature swine.

- Characterize the viremic profile of swine receiving the passive transfer of anti-JEV mAb JEV-31.
- Determine the severity of neurotropic disease in swine passively immunized with anti-JEV mAb JEV-31.

Specific aim 3: Determine the *in vivo* protection of an anti-EDI/EDII mAb in miniature swine.

- Characterize the viremic profile of swine receiving the passive transfer of anti-JEV mAb JEV-169.
- Determine the severity of neurotropic disease in swine passively immunized with anti-JEV mAb JEV-169.

To determine the vector competence of mosquito species in North America for USUV, three mosquito species were selected for testing because of their involvement in the transmission of WNV and SLEV, two related JE serocomplex flaviviruses in the New World. The susceptibility and vector competence of North American mosquitoes for USUV was investigated through the oral challenge of *Ae. albopictus, Cx. pipiens,* and *Cx. quinquefasciatus*. This will help to determine the likelihood that there could be further dispersal of USUV in the future and discover what could be target species for vector control in the event of a disease outbreak in North America.

To evaluate vaccine prevention platforms, a challenge-protection study was performed through the passive immunization of the vaccination derived anti-JEV mAbs in miniature swine, a vertebrate host species directly involved in the transmission of JEV in nature. Understanding

the suitability of miniature swine to study JEV is important as their use could provide results more aligned with human outcomes. The use of a natural amplification host to study JEV could also provide a means to look for effective methods to limit JEV replication in swine, thus severely restricting a natural reservoir of the disease. The protective efficacy of two vaccine derived anti-JEV mAbs recognizing different domains of the envelope protein in miniature swine were also characterized. This aimed to provide insight into the effectiveness of current JE vaccines against a GI strain in addition to investigating the potential of swine for these types of evaluations.

Overall, the results of the studies described in this dissertation can be directed at helping control and stop the establishment of JEV, USUV, or other flaviviruses into novel geographic locations. These data can be utilized in the design of risk assessment models and support future surveillance programs and control measures. This can aid in the control of mosquito species known to play a role in flavivirus transmission, as well as assist the development of new and improved flavivirus vaccines and therapeutics. As the geographic spread of flaviviruses is likely to continue in future years, the development of effective prevention measures is urgently needed to protect at-risk populations from neurological complications.

Chapter 2 - Materials and Methods

Introduction

This chapter will detail the materials and methods utilized in this dissertation. It will begin with describing methods for the oral challenge of *Aedes albopictus, Culex pipiens*, and *Culex quinquefasciatus* to determine the susceptibility and vector competence of North American mosquitoes for Usutu virus (USUV) with the results in chapter 3. The methods for the assessment of two anti-Japanese encephalitis virus (JEV) monoclonal antibodies (mAbs) in miniature swine will also be described in this chapter with the results provided in chapters 4 and 5.

Materials and Methods for North American Mosquito Competence for Usutu Virus

Cell lines

African green monkey Vero76 cells (CRL-1587) were maintained in L-15 media supplemented with 10% FBS, TPB, penicillin, streptomycin, and L-glutamine (Higgs et al., 2006). The cells were cultured at 37°C and used for titration of viral stocks using the $TCID_{50}$ method (Reed and Muench, 1938).

Virus

The prototype SAAr1776 strain was used for all oral (per os) infection of mosquitoes. The strain was originally isolated from a pool of *Culex neavei* in South Africa in 1959. Prior to the experiment, the strain obtained from the Division of Vector-Borne Diseases, Centers for Disease

Control was passaged seven times in suckling mouse brain and twice in Vero cells. Two additional passages in Vero76 cells were performed to generate stocks for per os (oral) challenge. Phylogenetically, the SAAr1776 strain forms a monophyletic cluster with recent isolates of USUV in Europe and has the closest relatedness to the sequence of USUV MB119/06 isolated in Spain in 2006 (Bakonyi et al., 2014).

Mosquitoes and *per os* infection

Colonies of *Cx. pipiens* (F21) and *Cx. quinquefasciatus* (F21) used in this study were established from larvae collected from Ewing Township, Mercer County, NJ and Vero Beach, FL in August 2015 (Huang et al., 2016a). The colony of *Ae. albopictus* (F4) tested in this study was derived from eggs collected from Trenton, Mercer County, NJ in July of 2016.

All mosquitoes were maintained with 20% sucrose solution under a 16-hour light: 8-hour dark photo regimen at 28˚C. Eight to ten-day old mosquitoes were deprived of sugar for 48 hours and water for 24 hours before per os infection. USUV stock and defibrinated sheep blood (Colorado Serum Company, CO) were mixed in equal volumes to produce artificial viremic blood meals. Blood meals were administered to *Culex* species mosquitoes in gallon size cartons through cotton pledgets at room temperature for 1 hour. Oral challenge of *Ae. albopictus* was performed with a Hemotek membrane feeding apparatus (Nuckols et al., 2015). Control groups received sterile defibrinated sheep blood mixed with tissue culture media.

Titers of viremic blood meals were determined by titration of remaining blood meals aspirated from cotton pledgets and individual blood feeders after the completion of each per os infection experiment. Fully engorged mosquitoes were collected under cold anesthetization and returned to the cartons. Engorged mosquitoes in each group were frozen immediately after

receiving blood meals to confirm ingestion of USUV and to quantify the viral titer of the infected blood ingested by mosquitoes. The titers of the viremic blood meals used for each experiment are listed in chapter 3.

Mosquitoes were sampled at 7- and 14-days post-infection (DPI) to characterize the infection process of USUV. To determine the incidence of disseminated infection, dissection of mosquitoes was performed to separate the abdomen from the head, wings, and legs (Figure 2-1). The abdomen contains the midgut where the infection of arboviruses is initially established. The head, wings, and legs are the secondary tissues, which are infected with viruses disseminated from the midgut. A second group of mosquitoes was collected, without dissection, to determine replication kinetics of USUV in whole mosquitoes. Mosquitoes (whole and dissected) were frozen in individual 2 mL Eppendorf tubes containing 2.8 mm metal beads and 96 µL L-15 media and 4 µL amphotericin.

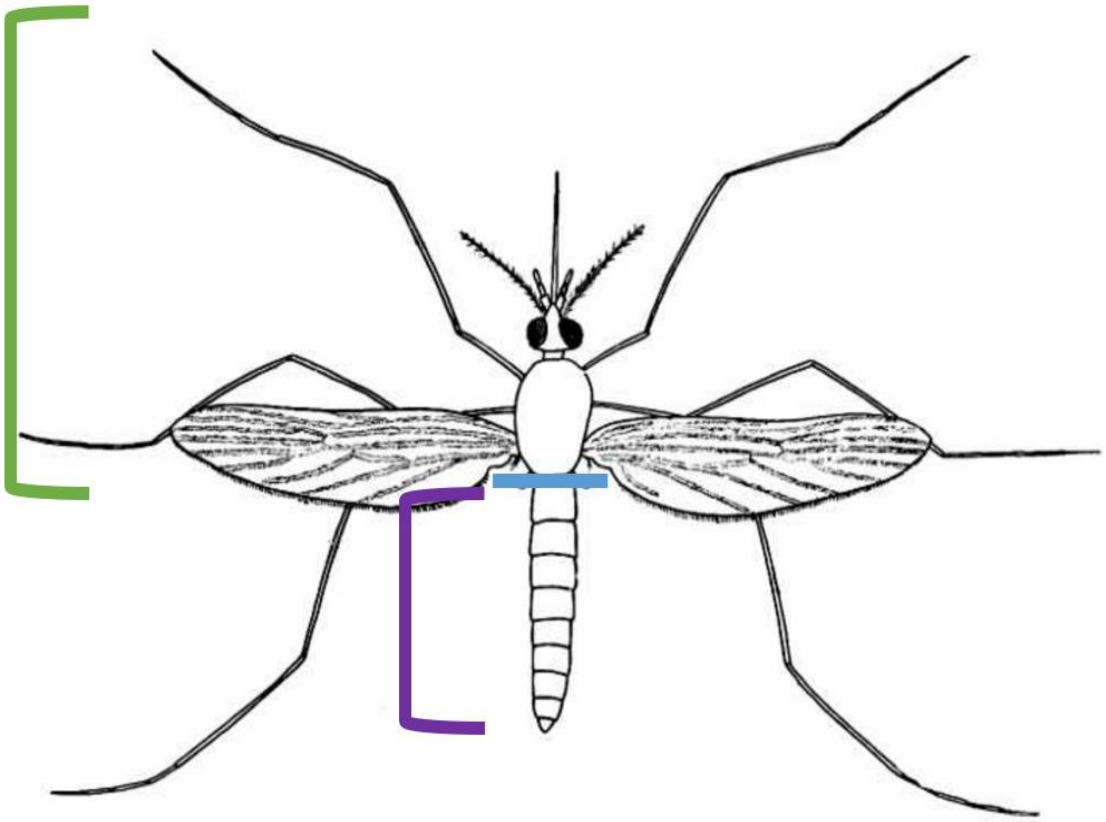

Figure 2-1. The dissection of mosquitoes. Dissected mosquitoes were separated at the blue line. This allowed for the abdomen containing the midgut (purple bracket) to be separated from the secondary tissues (green bracket). The secondary tissues contain the head, wings, and legs. Modified and used with permission from (Mackean, 2021).

Forced salivation

At 14 DPI, saliva of each mosquito was collected through forced salivation for 60 minutes (Huang et al., 2016b; Vanlandingham et al., 2004). Mosquitoes were cold anesthetized by putting cartons at four degrees Celsius until all mosquitoes were knocked down in the bottom of the carton. The mosquitoes were then transferred into a Petri dish with a lid. Using forceps and a scalpel the legs and wings were removed from the mosquito. Warm immersion oil was added to a 50 µL capillary tube by surface tension. The proboscis of the mosquitoes was placed into the top of the

capillary tube. After 60 minutes the mosquitoes were removed and stored in previously labeled tubes. The oil containing saliva from the capillary tube was expelled with pressure into 1.5 mL tube with 150 µL L-15 media.

Detection of Usutu virus in mosquito tissues

To quantify infectious viruses, all samples containing mosquito tissues were homogenized using a TissueLyser II apparatus (Qiagen, Germantown, MD) at 26 Hz for four minutes and titrated by 50% tissue culture infectious dose ($TCID_{50}$) method with Vero76 cells (Higgs et al., 2006). The mosquito samples were titrated in duplicate by adding 100 µL of undiluted sample followed by 10-fold serial dilutions up to 10^{10}-fold dilution. Each dilution was performed by transferring 10 µL into 90 µL of L-15 media. Negative control wells received fresh L-15 media. Each well of the 96-well plate received 100 µL of Vero76 cells. Plates were sealed with parafilm and incubated at 37°C for 7 days. Individual wells were evaluated for cytopathic effect visually after staining with 200 µL of amido black stain containing 1% amido black B10 suspended in 10% glacial acetic acid and 35% isopropanol aqueous solution at room temperature for at least 30 minutes. Each plate was washed with regular tap water before being read and titers were calculated using the Reed-Muench method (Reed & Muench, 1938).

Infection was calculated based on the overall percentage of infected mosquitoes among mosquitoes tested. Viral dissemination was demonstrated by the detection of USUV in the secondary tissues of infected mosquitoes. Dissemination was calculated as positive detection of infectious viruses in secondary tissues among dissected mosquitoes that were infected with USUV.

Extraction with QIAamp Viral RNA mini kit

A nested reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect the presence of USUV in the saliva of infected mosquitoes. The transmission rate of USUV was determined by the incidence of positive detection of viral RNA among infected mosquitoes. Viral RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Germantown, MD). Five hundred and sixty µl of Buffer AVL was pipet into 2 mL microcentrifuge tube. One hundred and forty µl of a saliva sample was added to the microcentrifuge tube and was vortexed for 15 seconds. The tubes were incubated at room temp for 10 minutes and then centrifuged briefly to remove drops from inside lid. Five hundred and sixty µl of ethanol was added and mixed by vortexing 15 seconds and briefly spun in the centrifuge. Six hundred and thirty µl of the solution from previous steps was added to a QIAamp mini column in a 2 mL collection tube. The cap was closed and then the tubes were centrifuged at 8000 rpm for one minute. The collection tubes were emptied, and the remainder of the mixture was added to the column. The column from the collection tube was removed and replaced with a clean collection tube. Five hundred µl of buffer AW1 was added and tubes were centrifuged for one minute at 8000 rpm. The column was removed from the collection tube and placed in a clean collection tube. Five hundred µl of AW2 was added and then tubes were centrifuged for three minutes at 13,000 rpm (maximum speed). The column was removed from the collection tube and placed in a clean tube. Sixty µl of molecular grade water was added then tubes were incubated at room temperature for one minute and centrifuged for one minute at 8000 rpm. The column was removed and discarded. Extractions were stored in RNAse free microcentrifuge tubes and kept at -80°C.

Detection of Usutu virus in saliva by nested polymerase chain reaction

Complementary DNA was produced by reverse transcription of Superscript III reverse transcriptase (Life Technologies, Carlsbad, CA) with the VD8 primer (5′-GGGTCTCCTCTAACCTCTAG-3′) that is broadly reactive to the 3′ untranslated region of all mosquito-borne flaviviruses (Pierre et al., 1994). The first amplicon was produced using Platinum Taq polymerase (Life Technologies, Carlsbad, CA) using the VD8 and EMF1 (5′-TGGATGACSACKGARGAYATG-3′) primer set (Figure 2-2) (Pierre et al., 1994). The EMF1 primer is degenerate with nucleotide codes R (adenine or guanine), S (guanine or cytosine), K (guanine or thymine), and Y (cytosine or thymine). The second amplicon was derived from the second RT-PCR reaction using two USUV specific primers, USUV 10242–10261 (5′-GCAACGTGGGCTGAAAACAT-3′) and USUV 10795–10776 (5′-AGTTCGCATCACCGTCTGTT-3′) (Figure 2-2). The USUV-specific primer set was designed using the Primer-BLAST algorithm available from the website of National Center of Biotechnology Information. All amplicons were separated and visualized by electrophoresis using 1% agarose gel. For generation of complementary DNA by reverse transcription 1 µL 10 µM VD8 primer,1 µL 10 mM dNTP mix, 3 µL H20, and 8 µL RNA were added. Tubes were heated for five minutes, then the thermocycler was paused, and the tubes were put on ice for one minute. Four µL 5x FS Buffer, 1 µL DTT, 1 µL RNAseOUT, and 1 µL Superscript III were added. For the first amplicon 2.5 µL of template cDNA, 2.5 µL 10X PCR buffer, 0.5 µL 10 mM dNTP mix, 0.75 µL $MgCl_2$, 0.5 µL 10 µM VD8 primer, 0.5 µL 10 µM EMF1 primer, 0.1 µL Taq polymerase, and 17.65 µL molecular grade water were added. For generation of the second amplicon 2.5 µL of template cDNA, 2.5 µL 10X PCR buffer, 0.5 µL 10 mM dNTP mix, 0.75 µL $MgCl_2$, 0.5 µL USUV (5′-GCAACGTGGGCTGAAAACAT-3′), 0.5 µL USUV (5′-

AGTTCGCATCACCGTCTGTT-3′), 0.1 µL Taq polymerase, and 17.65 µL molecular grade water were added. Reactions were performed with a Bio-Rad Thermal cycler (Bio-Rad, Hercules, CA) with cycling parameters for reverse transcription at 65°C for five minutes, ice for one minute while reactions were assembled, 50°C for 50 minutes, and 85°C for five minutes. For PCR the initial denaturation was two minutes at 94°C; denaturation for 30 seconds at 94°C; annealing 30 seconds at 49°C, extension for one minute at 72°C for 35 cycles and a final extension for five minutes at 72°C.

For creation of a 1% gel, one gram of agarose was measured. The agarose powder was mixed with 100 mL 1xTAE in microwavable flask and microwaved until the agarose was completely dissolved. When the agarose was cooled down it was poured into a gel tray with a well comb. The gel was allowed to completely solidify before samples were loaded. The agarose gel was placed into the electrophoresis unit and filled with 1xTAE until the gel was completely covered. A 100 base pair maker was loaded as well as the samples into the wells of the gel. When loading the gel 1 µL red marker/ 5 µL sample was added. The gel was run for 20 minutes at 125 volts. The power was then turned off, electrodes removed, and the gel was removed from box. Using UV light, the bands were observed.

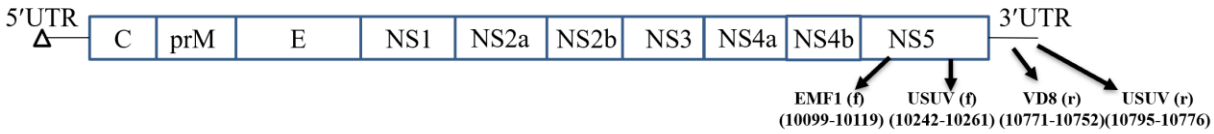

Figure 2-2. Primers used for nested polymerase chain reaction. The primers target the NS5 and 3′ UTR that are both considered highly conserved regions of the genome.

Statistical analysis

GraphPad Prism (version 8.1.1) was used for statistical analysis. The infection, dissemination, and transmission rates of USUV in infected mosquitoes were analyzed using Fisher's exact test. Fisher's exact test can be applied to evaluate categorical data to determine the significance of association. It compares the two data sets in a 2x2 contingency table.

Materials and Methods for the Evaluation of the Protective Efficacy of Two Anti-Japanese Encephalitis Virus Monoclonal Antibodies in Miniature Swine

Cell lines

African green monkey Vero76 cells (CRL-1587) were maintained in L-15 media supplemented with 10% FBS, TPB, penicillin, streptomycin, and L-glutamine (Higgs et al., 2006). The cells were cultured at 37°C and used for titration of viral stocks using the $TCID_{50}$ method (Reed and Muench, 1938).

Aedes albopictus C6/36 cells (CRL-1660) (source: Arthropod-Borne Animal Disease Research Unit, Agriculture Research Service, United States Department of Agriculture) were maintained in L-15 media (Life Technologies, Carlsbad, CA) supplemented with heat-inactivated 10% fetal bovine serum (FBS), tryptose phosphate broth (TPB), penicillin, streptomycin, and L-glutamine (Huang et al., 2016b). The cells were incubated at 28°C and used for the propagation of all stock viruses.

Virus

JEV strain JE-91 (Genbank access number GQ415355.1), a GI-b isolate originally from mosquitoes collected in Korea, was propagated in *Ae. albopictus* C6/36 cells for five days prior to collection and maintained in Leibovitz-15 (L-15) media. The virus stocks, acquired from existing virus culture collection in the laboratory of Dr. Alan D. T. Barrett, University of Texas Medical Branch, were harvested, and the supernatant was then aliquoted and stored at -80°C until use (Schuh et al., 2010). Prior to the experiment, the virus was passaged once in Vero cells and once in C6/36 cells to mimic the mammal to vector cycle of the virus in nature. Infectious virus titers of the stocks were determined by $TCID_{50}$ method and determined to be 3.3×10^7 $TCID_{50}$/mL (Higgs et al., 2006).

Monoclonal antibodies

Three murine IgG2c mAbs, anti-JEV JEV-31 and JEV-169 mAbs, and anti-chikungunya virus (CHIKV) CHK-152 mAb, were provided by Dr. Michael S. Diamond at Washington University, St. Louis, MO and produced and purified by Bio X Cell (West Lebanon, NH) (Fernandez et al., 2018). Both JEV-31 and JEV-169 were generated from C57BL/6 mice deficient for interferon regulatory factor 3 that were infected and boosted with JEV GIII vaccine strain SA-14-14-2. Neutralization assays were preformed and demonstrated JEV-31 and JEV-169 neutralize strains of GI, GII, GIII, and GIV *in vitro*. Site-directed mutagenesis and hydrogen-deuterium exchange mass spectrometry identified sites within EDI/EDII (JEV-169) and EDIII (JEV-31) as key epitopes for neutralization. The epitope mapping completed by Fernandez et al. allowed for the selection of two broadly neutralizing mAbs against JEV recognizing different domains of the E protein to be used in this work. The CHK-152 mAb was originally generated and evaluated by

Pal et al. 2013. The CHK-152 mAbs were utilized as an isotype control as it allowed for antibody characteristics to be matched without antibody specificity for the target antigen (JEV). Briefly, interferon deficient mice were infected and boosted with CHIKV. Neutralization tests determined CHK-152 was strongly neutralizing *in vitro* and protected mice from disease caused by CHIKV.

Animal experiment and design

Two anti-JEV mAbs were passively transferred and evaluated for their protective efficacy in miniature swine. This was completed under BSL-3AG conditions at the Biosecurity Research Institute at Kansas State University (KSU). The KSU Institutional Animal Care and Use Committee approved the following experimental procedures and animal use. All methods were carried out in accordance with approved protocols and regulations.

Nineteen three-week-old Sinclair Yucatan pigs (Sinclair Biosciences, Columbia, MO) were housed in BSL3-Ag conditions at the Biosecurity Research Institute at KSU, Manhattan, KS. Pigs were chosen for this animal experiment, as they are amplifying hosts that develop viremia and sustain the transmission of JEV in nature. Pigs infected with JE can develop neurotropic disease similar to human infection, and the related pathological outcomes in the two species allow for the use of swine to characterize immune protection to JEV infection and determine the efficacy of candidate vaccines and antiviral treatments. The Yucatan pig was selected due to comparable immunization and experimental challenge regimens that were previously performed in the same species (Park et al., 2018).

Upon arrival, the pigs were randomly assigned to a treatment group. Two pigs were used as negative controls receiving sterile saline in lieu of mAbs and JEV challenge. Two pigs were given either mAb JEV-31 or mAb JEV-169 and challenged with sterile saline as the mAb in order

to assess the safety of immunizing pigs with mouse mAbs. The five animals in the non-immune isotype control group were given CHK-152 mAbs and challenged with JEV JE-91 to allow for antibody characteristics to be matched without antibody specificity for the target antigen (JEV). Five pigs were assigned to the mAb JEV-31 group and five pigs were in the mAb JEV-169 group and challenged with the JE-91 strain.

The groups of pigs were kept in separate pens to avoid potential non-vector transmission and allowed to acclimate for five days. The pigs were weighed several times prior to passive immunization and the average weight of the pigs prior to passive immunization was five kg. After the five-day acclimation period, the pigs were passively immunized intramuscularly (IM) based on their group. Passive immunization was administered through the IM route with a 23-gauge, 1-inch needle in one of the limbs of the pig. Each site received no more than 1 mL of mAb with a maximum of four sites utilized per animal. Twenty-four hours after passive immunization, the pigs had their blood drawn before they were inoculated intradermally (ID) in the ear with either 100 µL of sterile saline or JE-91 JEV strain at 10^7 $TCID_{50}$/ml to mimic natural infection from an infected arthropod vector. The animals were monitored daily for clinical signs and pain. Temperatures were taken daily as well as blood samples, nasal swabs, and oral fluid collection. Three days post infection fecal swabs were also taken, and all animals were euthanized.

Sample collection

The samples were selected based on the tissue tropism of JEV in experimentally challenged swine (Park et al., 2018; Ricklin et al., 2016b; Yamada et al., 2004). Pen-based oral fluid and nasal swabs were collected to evaluate oronasal shedding. Serum was collected to evaluate viremia. Fecal swabs were collected to assess viral shedding through the digestive tract. Evaluated

lymphoid tissues included the thymus, tonsils, medial retropharyngeal lymph node, mesenteric lymph node, medial iliac lymph node, and spleen. The lymphoid tissues were evaluated to assess systemic infection. Selected tissues of the central nervous system were the cerebellum, cerebrum (frontal and parietal lobes), olfactory neuroepithelium, and sciatic nerve to evaluate neuroinvasion.

Serum collection

Whole blood was collected from pigs via the right external jugular vein into red-top vacutainer tubes at zero to three DPI. The jugular vein was selected for sample collection as it is safer to access than the cranial vena cava and can be easily accessible in young pigs. The right side of the jugular vein was used to avoid any damage to the phrenic nerve. Tubes were centrifuged at 2,000 x g for 10 minutes. The serum was removed from the top of the blood clot and aliquoted into 2 mL microcentrifuge tubes and stored at -80°C.

Oral fluid collection

Oral fluids were collected daily from each pen of animals using pre-packaged oral fluid collection kits with previously described by Lyons et al. (Lyons et al., 2018). Cotton rope 1.25 cm in diameter was tied to a metal bar on the side of the pen at pig shoulder height. The rope was left in the pen for the pigs to chew for approximately twenty minutes or until the rope was saturated. The ropes were then placed in plastic bags attached to 15 mL conical tubes and oral fluid was extracted by manually squeezing the fluid into the tubes. Oral fluids were transferred from the conical tubes into 1.5 mL microcentrifuge tubes and stored at -80°C.

Nasal swab collection

Individual nasal swabs were obtained daily with sterile cotton swabs. The side of the nares for the swab was alternated each day. The swabs were then placed into 1 mL of L-15 media and agitated by hand twirling the swab in the media filled tube. The samples were then vortexed and stored at -80°C.

Fecal swab collection

Fecal swabs were collected on day three. Sterile cotton swabs were used to collect fecal remnants from the anus of all the pigs. The swabs were then placed in labeled 15 mL conical tubes that contained 1ml of L-15 media and agitated by hand twirling the swab in the media filled tube. The conical tubes were vortexed and stored at -80°C.

Tissue collection

At necropsy (3 DPI), approximately a five mm^3 square of tissue was collected and placed into 1 mL of L-15 media. The samples were stored at -80°C prior to analysis. The tissues were selected based on previous analyses determining the dissemination and tissue tropism of JEV (Park et al., 2018). Tissues were weighed before being homogenized to evaluate the tissue viral loads. The tissues were homogenized using the TissueLyser II system (Qiagen) at 26 Hz for four minutes followed by centrifugation at 10,000 × g for 10 minutes.

Determination of Japanese encephalitis virus infectious titers

The quantitative reverse transcription PCR (RT-qPCR) assay utilized for the determination of infectious titers was previously evaluated using samples collected from pigs experimentally

challenged with the JE-91 strain of JEV (Lyons et al., 2018). To create standard curves for RT-qPCR assays and for challenge utilization, titers of viral stock was determined using $TCID_{50}$. Stock virus was titrated in duplicate by inoculating 100 µL of undiluted virus followed by 10-fold serial dilutions up to 10^{10}-fold dilution. Each dilution was performed by transferring 10 µL inoculum into 90 µL of L-15 media. Negative control wells received fresh L-15 media. Each well of a 96-well plate received 100 µL of Vero76 cells. Plates were sealed with parafilm and incubated at 37°C for 7 days. Individual wells were evaluated for cytopathic effect visually after staining with 200 µL of amido black stain containing 1% amido black B10 suspended in 10% glacial acetic acid and 35% isopropanol aqueous solution at room temperature for at least 30 minutes. Each plate was washed with regular tap water before being read and titers were calculated using the Reed-Muench method (Reed and Muench, 1938).

Extraction of viral nucleic acid and reverse transcriptase-quantitative polymerase chain reaction

Genome equivalents were determined using a previously published TaqMan one-step RT-qPCR assay targeting the genomic fragment encoding the nonstructural protein five (Lyons et al., 2018). The RNA was extracted from serum, tissue, nasal, oral, and fecal samples with Trizol LS (Invitrogen) and Directzol Miniprep Plus kit (Zymo, Irvine, CA) according to manufacturer's recommendations as follows:

Extraction of RNA with Directzol MiniPrep Plus kits

Two hundred and fifty µL of sample and 750 µL Trizol LS reagent were mixed in a 2 mL microcentrifuge tube. The tubes were incubated at room temperature for 10 minutes to ensure

complete lysis and dissociation of ribonucleocomplex. An equal volume of absolute ethanol (1,000µL) was added to the sample lysed in Trizol and mixed by inverting the tube. The tubes were briefly spun down at 10,000 r.p.m. to remove the fluid in the lid of each tube to avoid cross-contamination. The lysates were loaded and flowed through the Zymo Spin Column by repeated spinning at 13,000 g for 30 seconds. Four hundred µL of RNA Wash Buffer was added to the column and centrifuged at 13,000 g for 30 seconds. In RNase-free tube 5 µL DNase I and 75 µL of DNA digestion buffer was added and mixed; this was added directly to column matrix and allowed to incubate at room temperature for 15 minutes. Four hundred µL Direct-zol RNA PreWash was added to the column and centrifuged at 13,000 g for 30 seconds. The flow through was discarded. Four hundred µL Direct-zol RNA PreWash was added to the column again and centrifuged at 13,000 g for 30 seconds with the flow through discarded. Seven hundred µL of RNA Wash Buffer was added to the column and centrifuged at 13,000 g for two minutes to ensure complete removal of wash buffer. An additional spin at 13,000 g for two minutes was done before the transfer into an RNase-free tube. To elute the RNA 25 µL of DNase/RNase-free water was added directly to the column and centrifuged at 13,000 g. The elution step was repeated and then the eluted RNA was aliquoted into 1.5 mL microcentrifuge tubes and stored at -80°C.

The RT-qPCR assay performed using iTaq Universal Probes One-step kit

The RT-qPCR assay was selected based on previous studies by Lyons et al. in 2018. The assay targets the conserved nucleotide sequences in the NS5 gene and detects presence of JEV through the synthesis of amplicons between nucleotide positions 10,224 and 10,286, between the 3′ end of NS5 gene and 5′ end of the 3′ UTR of the JEV genome (Table 2-1) (Pyke et al., 2004). For each reaction 4 µL of RNA extract, 10 µL iTaq universal probe reaction mix, 0.5 µL iScript

advanced reverse transcriptase, 1 µL forward primer (5′ATCTGGTGYGGYAGTCTCA3′), 1 µL reverse primer (5′CGCGTAGATGTTCTCAGCCC3′), 0.4 µL probe (5′CGGAACGCGATCCAGGGCAA3′), and 3.1 µL molecular grade water were added. The reactions were performed on the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with cycling parameters of reverse transcription for 30 minutes at 50°C, initial denaturation for 10 minutes at 95°C, denaturation for 15 seconds at 95°C, and annealing and extension for one minute at 60°C for 40 cycles.

For each reaction, a standard curve was generated by 10-fold serial dilution of RNA extract derived from a JEV stock of known titer at 5.2 x 10^6 $TCID_{50}$/mL (Figure 2-3). The limit of detection was 20.3 $TCID_{50}$/mL. Results were reported as genome equivalent to $TCID_{50}$/mL. Samples were considered positive when the Cq value was less than 33.

Table 2-1. Sequence and location within in the Japanese encephalitis virus genome of the primers and probe. Y represents a degenerate base.

	Genomic Sequence	Genomic Position
Forward primer	ATCTGGTGYGGYAGTCTCA	10224-10242
Reverse primer	CGCGTAGATGTTCTCAGCCC	10267-10286
Probe	CGGAACGCGATCCAGGGCAA	10244-10263

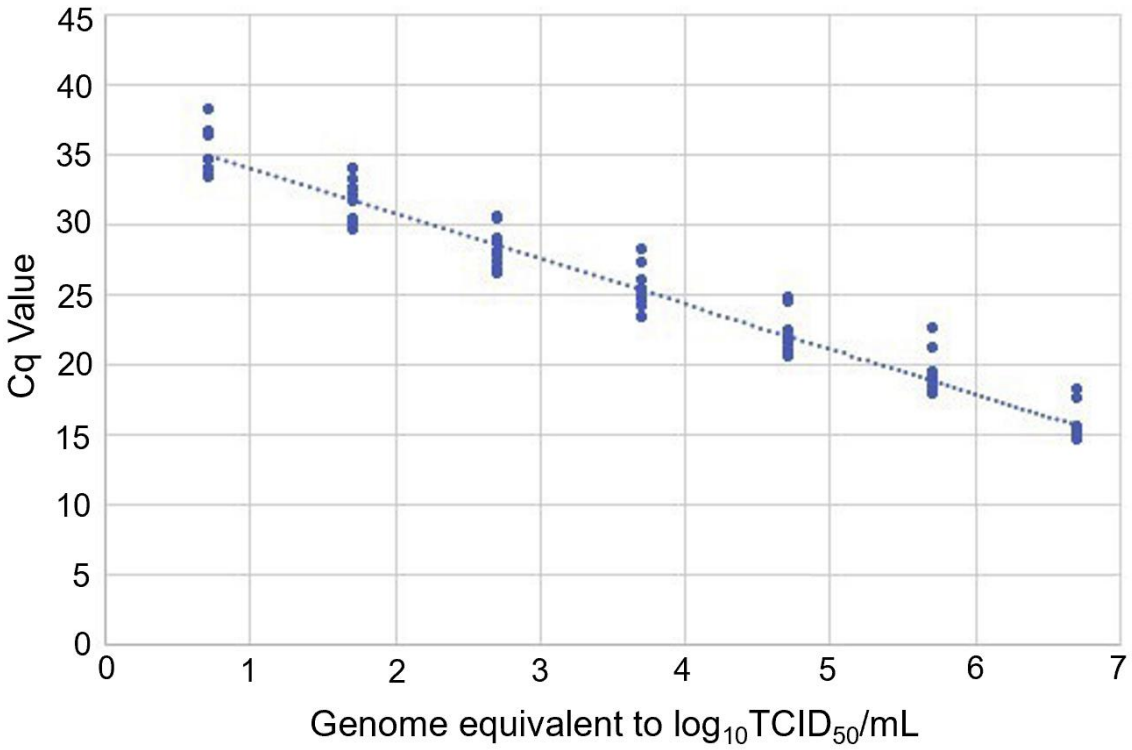

Figure 2-3. Standard curve of the RT-qPCR assay. Y= -1.392ln(x) + 37.193, R^2 = 0.949. Limit of detection was estimated based on the trend line at Cq value of 33.

Plaque reduction neutralization test

Neutralizing antibody titers of day zero serum samples were determined by performing plaque reduction neutralization tests (PRNT). The 24 well plates were seeded with Vero76 cells and left in the 37 °C incubator overnight. Serum samples were heat inactivated at 56 °C for 30 minutes in a water before being diluted between 1:10 and 1:320. Ninety µL of L15 media was added to 10 µL of serum. To dilute, 50 µL was serially pipet with the last 50 µL discarded. To each concentration of serum 50 µL (approximately 30 PFU) of JE-91 strain was added and then incubated for one hour at 37 °C. The wells of the plates had approximately 1 mL of media removed, ensuring to leave enough supernatant that wells would not dry out. Next 50 µL of virus-serum mixture were added into the appropriate wells. Plates were kept in 37 °C incubator for one

hour, rocked back and forth and side to side every 15 minutes to allow absorption and 2.5 mL of overlay with 1% methylcellulose added. After five days of incubation at 37 °C, plates were fixed and stained with 10% formalin/PBS and crystal violet stain. Plaques were counted, and neutralizing antibody titers were calculated based on a 50% or greater reduction in plaque counts ($PRNT_{50}$).

Generation of crystal structures

PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC, was used for graphical display of crystal structures.

Statistical analyses

GraphPad Prism (version 8.1.1 GraphPad Software, Inc., LaJolla, CA) was used for statistical analyses. Numerical, quantitative data can be continuous, measurements that are uncountably infinite (Vetter, 2017). Data that follow a normal distribution have a symmetric and unimodal shape called bell-shaped distribution. The Shapiro-Wilk test can be used to test distribution. If the data does not follow normal distribution nonparametric tests can be used because they do not assume that the data fits a normal distribution. The Kruskal Wallis analysis of variance (ANOVA) is the nonparametric alternative to the one-way ANOVA to determine if two or more groups are different and the Mann Whitney U test compares two groups. While ANOVA tests can determine if there is a significance difference between groups, it does not say which groups are different; this would require a post hoc test such as the Dunnett's test. Dunnett's test compares the means from experimental groups against a control group to see if there is a difference.

The data that is plotted with a box and whisker box plot have a box from the 25th to 75th percentiles. The line in the middle of the box is plotted at the median. The whiskers of the box plot show the minimum and maximum values, and each individual data point is shown on the graph.

Continuous data that follow normal distribution

Normality of the data was assessed with the Shapiro-Wilk test. The cerebellum data differed from other tissues, as the viral loads in this tissue were normally distributed; thus, the cerebellum was analyzed using ANOVA and the Dunnett's test with the CHK-152 mAb pigs assigned as the control group.

Continuous data that do not follow normal distribution

All data besides the cerebellum did not follow normal distribution. Therefore, they were evaluated with nonparametric tests that do not assume the data fits a specific distribution type. The data were compared using the non-parametric Kruskal-Wallis ANOVA test to evaluate the degree of association between samples, if significant this was followed by Dunnett's test comparing treatment groups against the control group (the CHK-152 mAb pigs).

Chapter 3 - North American *Culex pipiens* and *Culex quinquefasciatus* are Competent Vectors for Usutu Virus

This chapter is adapted from a manuscript published in PLOS Neglected Tropical Diseases:

Cook, C. L.*, Huang, Y. S., Lyons, A. C., Alto, B. W., Unlu, I., Higgs, S., & Vanlandingham, D. L. (2018). North American *Culex pipiens* and *Culex quinquefasciatus* are competent vectors for Usutu virus. *PLoS neglected tropical diseases*, *12*(8), e0006732. *Published under maiden name. https://doi.org/10.1371/journal.pntd.0006732

Introduction

Usutu virus (USUV) is a mosquito-borne flavivirus classified under the Japanese encephalitis (JE) serocomplex in the *Flaviviridae* family. It was originally isolated in 1959 in South Africa (Williams et al., 1964). The earliest dispersal of USUV from the African continent was documented through the detection of viral RNA in tissues of the common European blackbird, *Turdus merula*, in Italy in 1996 (Weissenböck et al., 2013). Since 2001, the emergence of USUV has been continuously reported throughout Europe and has been responsible for the deaths of thousands of birds with an estimated 15.7% blackbird population decline attributed to the virus (Ashraf et al., 2015; Luhken et al., 2017). Infections with USUV have become a potential public health threat after reports of neurotropic diseases in humans linked to this virus.

Exposures to USUV typically lead to mild diseases and seroconversion in incidental hosts as observed in humans and horses (Barbic et al., 2013). Historically, human USUV infections have been primarily associated with self-limiting febrile illness and rash followed by the

development of neutralizing antibodies. Infection with USUV leading to neurotropic disease was only documented in immunocompromised individuals (Cavrini et al., 2009; Pecorari et al., 2009). However, more recent evidence indicates that severe disease could also take place in healthy adults which has considerably increased the public health significance of USUV (Grottola et al., 2017; Santini et al., 2015).

Similar to other members of the JE serocomplex, maintenance of USUV in nature depends on the enzootic cycle among viremic avian species as amplification hosts and the insect vectors able to carry and spread the disease. Transmission of USUV in Africa has been demonstrated to be vectored by ornithophilic mosquito species such as *Culex neavei* with additional isolates from pools of *Cx. pipiens*, *Cx. univitattus*, and *Coquillettidia aurites* (Mossel et al., 2017; Nikolay et al., 2012; Ochieng et al., 2013; Williams et al., 1964). Although isolates of USUV have been made from various zoophilic mosquitoes including *Cx. pipiens*, *Cx. modestus*, *Aedes albopictus*, and *Anopheles maculipennis* in Europe since 2001, vector competence studies suggest that USUV is mainly transmitted by competent *Cx. pipiens* populations (Busquets et al., 2008; Calzolari et al., 2013; Fros et al., 2015; Jost et al., 2011; Puggioli et al., 2017; Rudolf et al., 2015). This is likely due to the ornithophilic (bird feeding) nature of *Cx. pipiens* and their ability to thrive in domestic settings feeding on mammals including humans (Farajollahi et al., 2011).

As historically observed with the dispersal of multiple flaviviruses in the JE serocomplex, enzootic transmission can be quickly established in the presence of competent vectors and amplification hosts. The establishment of a JE serocomplex member to a novel geographic location was observed with the entry and spread of West Nile virus (WNV) to North America in 1999 with competent amplification hosts in birds, particularly American blackbirds, and competent vectors, primarily *Culex* species mosquitoes. The emergence of USUV in Europe during the last

two decades has significantly increased the likelihood of its further dispersal into other geographic regions including the United States. In this study, oral challenge with USUV was performed in North American *Ae. albopictus*, *Cx. pipiens*, and *Cx. quinquefasciatus* to determine their vector competence for USUV. The three species were selected based on their known involvement in the transmission of WNV, a flavivirus within the JE serocomplex, in North America. *Culex pipiens* and *Cx. quinquefasciatus* have been implicated as major vectors of WNV due to their vector competence, local abundance, and feeding preferences (Andreadis, 2012). *Aedes albopictus* have also been implicated as competent vectors for WNV as bridge vectors feeding on birds and humans abundant in urbanized areas (Rochlin et al., 2019).

Results

Infectious viruses of USUV isolated from orally challenged *Cx. pipiens* and *Cx. quinquefasciatus*, while *Ae. albopictus* appears unreceptive to infection.

Summarized in Table 3-1 are the average titers of the viremic blood meals. The engorgement of the viremic bloodmeal led to USUV being detected in all three mosquito species immediately after engorgement (Figure 3-1). Engorgement of infectious viruses led to establishment of infection in both *Cx. pipiens* and *Cx. quinquefasciatus* at 7- and 14-days post infection (DPI) (Figure 3-2 and Figure 3-3). Replication of USUV led to significant increases in the infection rates of *Cx. pipiens* from 25% (4/16) at 7 DPI to 58.6% (17/29) at 14 DPI ($p = 0.03$) and disseminated infection at 14 DPI (Table 3-2). There was no significant difference in the infection rates of USUV in *Cx. quinquefasciatus* at 7 (93.3%, 14/15) and 14 (70.0%, 21/30) DPI. Similarly, the dissemination rate of USUV in *Cx. quinquefasciatus* was not significantly different at 7 (66.7%, 4/6) and 14 (35.7%, 5/14) DPI. Despite *Ae. albopictus* being challenged with similar

titers of infectious virus as ingested by *Cx. pipiens*, *Ae. albopictus* were highly refractory to USUV infection, with no detection of virus at 7 (0.0%, 0/22) or 14 (0.0%, 0/27) DPI

Table 3-1. Summary of titers of viremic blood meals.

Mosquitoes Tested	**Average Titer of Viremic Blood Meals**
Cx. pipiens	7.5
Cx. quinquefasciatus	6.9
Ae. albopictus	5.9 ± 0.6

Titers listed in $\log_{10}TCID_{50}$/mL

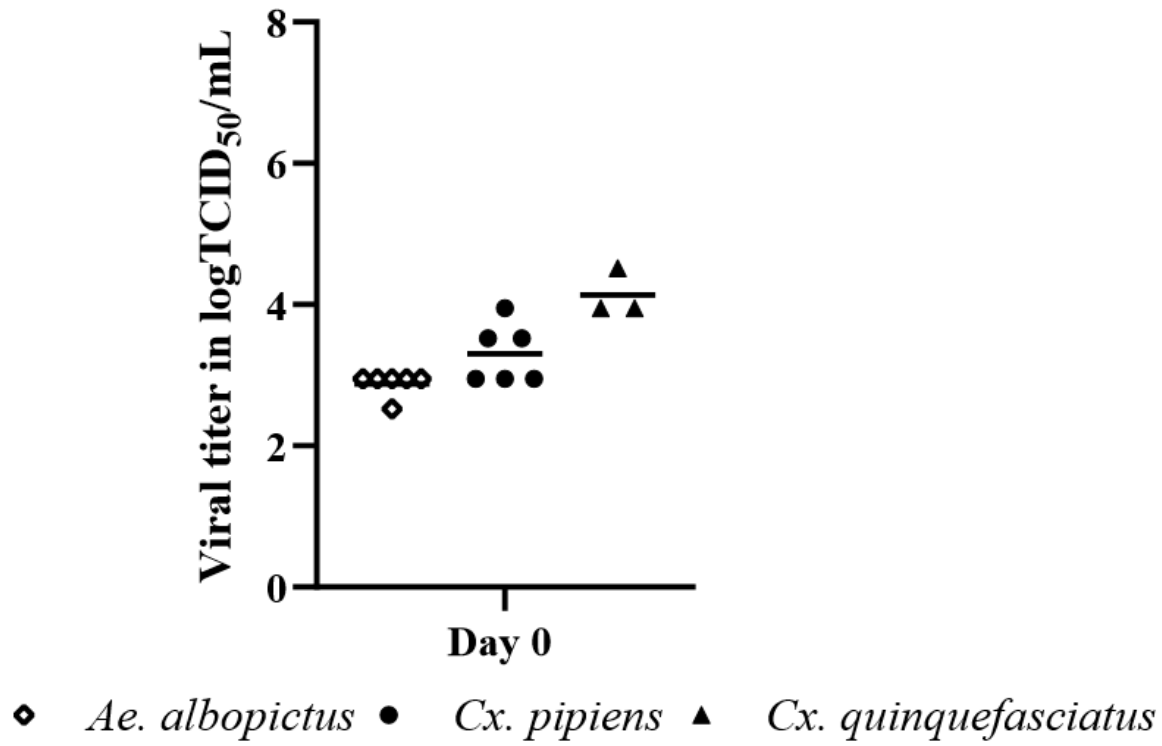

Figure 3-1. Infectious titers of whole mosquitoes infected with Usutu virus immediately after engorgement of viremic blood meals. Titers of individual infected *Aedes albopictus*, *Culex pipiens*, and *Culex quinquefasciatus* are shown in diamonds, circles, and triangles, respectively. The solid black line represents the mean titer of each species at 0 days post infection. Differences in titers of whole mosquitoes were analyzed using Kruskal Wallis ANOVA.

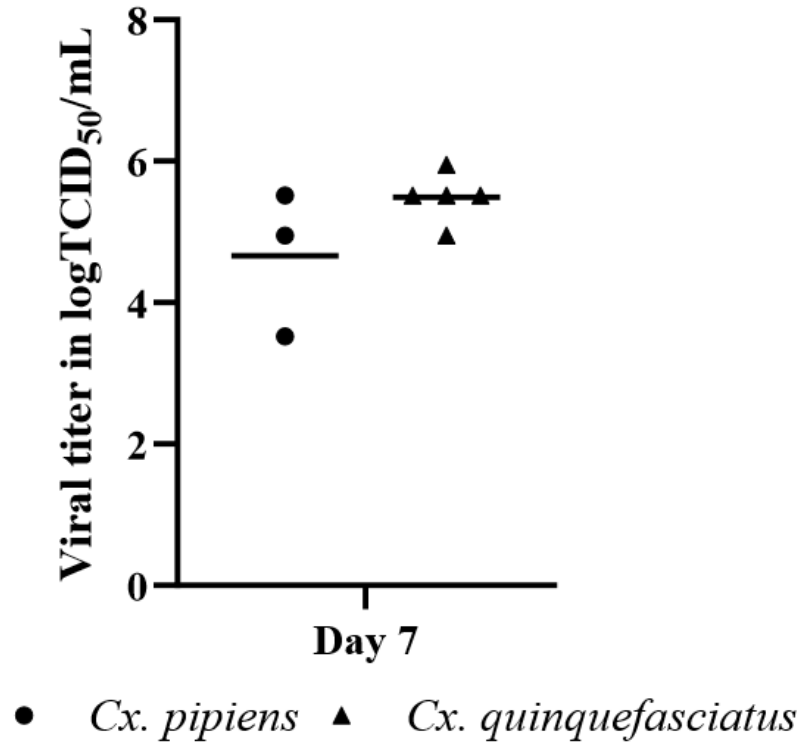

Figure 3-2. Infectious titers of whole mosquitoes infected with Usutu virus 7 days post infection. Titers of individual infected *Culex pipiens* and *Culex quinquefasciatus* are shown as circles and triangles, respectively. The solid black line represents the mean titer of each species at 7 days post infection. Differences in titers of whole mosquitoes were analyzed using the Mann Whitney U test.

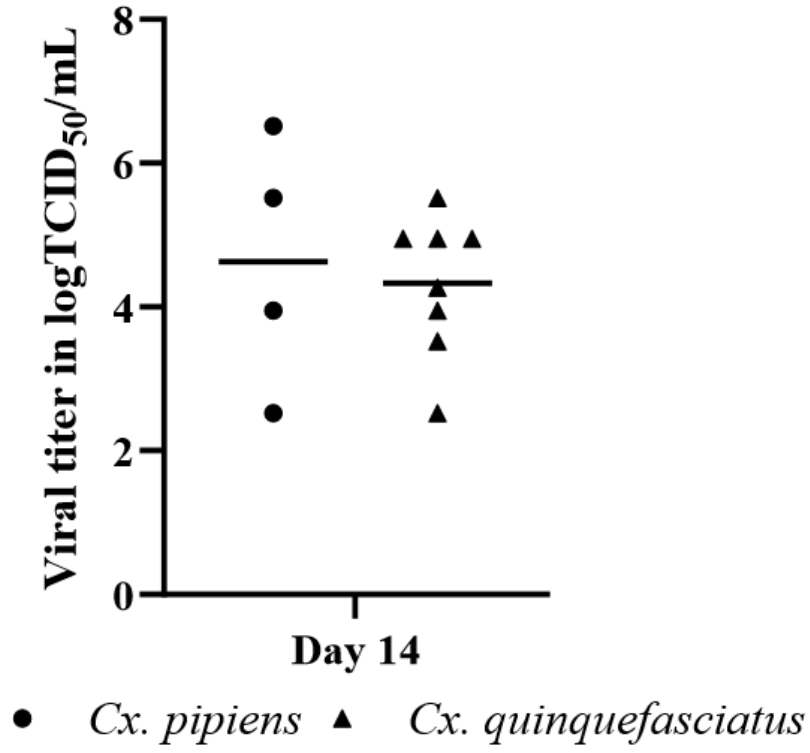

Figure 3-3. Infectious titers of whole mosquitoes infected with Usutu virus 14 days post infection. Titers of individual infected *Culex pipiens* and *Culex quinquefasciatus* are shown as circles and triangles, respectively. The solid black line represents the mean titer of each species at 14 days post infection. Differences in titers of whole mosquitoes were analyzed using the Mann Whitney U test.

Detection of viral RNA in saliva of infected mosquitoes

The detection of infectious viruses in *Cx. pipiens* and *Cx. quinquefasciatus* indicated that both species are susceptible to USUV through oral exposure. To evaluate potential for transmission by both species, nested RT-PCR was used to detect viral RNA in the saliva. Among infected *Cx. pipiens*, 23.5% (4/17) of saliva samples collected at 14 DPI were positive for the presence of USUV viral RNA (Table 3-2). Similarly, 19.0% (4/21) of infected *Cx. quinquefasciatus* had positive detection of viral RNA in saliva at 14 DPI

Table 3-2. Infection, dissemination, and positive detection in saliva of Usutu virus.

Mosquitoes Tested	Infection		Dissemination		Saliva
	7 DPI	14 DPI	7 DPI	14 DPI	14 DPI
Cx. pipiens	25% (4/16)	58.6% (17/29) *	100% (1/1)	92.3% (12/13)	23.5% (4/17)
Cx. quinquefasciatus	93.3% (14/15)	70% (21/30)	66.7% (4/6)	35.7% (5/14)	19.0% (4/21)
Ae. albopictus	0.0% (0/22)	0.0 % (0/27)	N/A	N/A	N/A

N/A = not applicable
* = significant difference
The infection, dissemination, and transmission rates of USUV in infected mosquitoes were analyzed using Fisher's exact test. Fisher's exact test can be applied to evaluate categorical data to determine the significance of association. It compares data sets in a 2x2 contingency table.

Discussion

The results demonstrate that North American *Cx. pipiens* and *Cx. quinquefasciatus* are susceptible to USUV and competent for its transmission. With the evidence demonstrating the vector competence of *Cx. pipiens* for USUV and its involvement of transmission in nature, the species is likely to be a critical player for the further dispersal of USUV in the future and a target species for vector control in the event of disease outbreak in North America. While there was no significant difference in infectious titers of whole mosquitoes collected at 0, 7, and 14 DPI, a minor increase in the infectious titer and the significant increase in infection rates observed in *Cx. pipiens* indicate that the species is permissive for the replication of USUV. Similarly, *Cx. quinquefasciatus* tested in this study also allowed the replication of USUV as demonstrated with maintenance of infectious titers of whole mosquitoes at 7 and 14 DPI. The replication of USUV in both species ultimately led to its dissemination from the midgut into secondary tissues including salivary glands, as demonstrated by the detection of viral RNA in the saliva of infected mosquitoes at 14 DPI Similarly, *Cx. pipiens* and *Cx. quinquefasciatus* have been determined to be competent laboratory vectors for Japanese encephalitis virus (JEV), St. Louis encephalitis virus (SLEV), and WNV (de Wispelaere et al., 2017; Huang et al., 2015; Turell, 2012). European *Cx. pipiens* and North American *Cx. quinquefasciatus* demonstrated they could be competent vectors for JEV (de Wispelaere et al., 2017; Huang et al., 2015). Similarly, both *Cx. pipiens* and Cx. quinquefasciatus are susceptible to infection and dissemination with SLEV and WNV (Turell, 2012).

Although *Ae. albopictus* have been demonstrated to be competent for the transmission of multiple flaviviruses in the JE serocomplex, the species has been demonstrated to be highly refractory to USUV based on these and others' findings (Nikolay, 2015; Puggioli et al., 2017; Vanlandingham et al., 2016). As USUV is unable to establish infection in orally exposed *Ae.*

albopictus, the species is unlikely to be involved in the transmission and maintenance of USUV. Isolation of infectious viruses or detection of viral RNA from *Ae. albopictus* in nature may be a consequence of recent engorgement from viremic avian species as some *Ae. albopictus* populations have been demonstrated to have opportunistic feeding behaviors and utilize avian species as a source of blood meals (Richards et al., 2006).

Despite the known genetic differences between members of the *Cx. pipiens* complex in the Old and New World and differing methods for detection of USUV, *Cx. pipiens* and related species remain as the most probable vector for USUV in North America due to their mixed feeding preferences and adaptability to human environments, as well as their role in the transmission of JE serocomplex members WNV and SLEV (Fonseca et al., 2004). Notwithstanding the variance in methods, the results are comparable with the previous publication on the vector competence of populations of *Cx. pipiens* in Northwestern Europe for USUV (Fros et al., 2015).

These observations also do not exclude the possibility that other mosquito species can potentially serve as competent vectors for the transmission of USUV outside its endemic regions. This could potentially include *Cx. tarsalis*, a competent vector for SLEV and WNV in the United States (Goddard et al., 2002; Hammon and Reeves, 1943). As observed with Japanese encephalitis virus and WNV, dispersal of flaviviruses under the JE serocomplex often involves transmission by multiple mosquito species in nature. This strategy may ultimately increase the likelihood for the permanent establishment of enzootic transmission cycles and challenge the efforts in the formulation of vector control strategies. While there have been at least two JE serocomplex members present in the New World including WNV and SLEV, USUV remains likely to be established and co-circulate with related viruses in the potential event of its introduction. As co-circulation of USUV and WNV have been reported in Europe, it is highly unlikely that the potential

introduction of USUV will lead to the displacement of WNV (Nikolay, 2015). Whether or not co-circulation can ultimately result in the displacement of SLEV, as observed with the displacement of SLEV by WNV in California in 2003, remains unclear.

Chapter 4 - Protective Efficacy of Vaccination-Derived Anti-Japanese Encephalitis Virus Monoclonal Antibody JEV-31 in Miniature Swine

This chapter is adapted from a manuscript published in Antiviral Research:

Young, C. L., Lyons, A. C., Hsu, W.-W., Vanlandingham, D. L., Park, S. L., Bilyeu, A. N., Ayers, V.B., Hettenbach, S.M., Zelenka, A.M., Cool, K.C., Peterson, G.J., Higgs, H., Huang, Y.-J. S. (2020). Protection of swine by potent neutralizing anti-Japanese encephalitis virus monoclonal antibodies derived from vaccination. Antiviral Research, 174, 104675. doi: https://doi.org/10.1016/j.antiviral.2019.104675

* Data displayed in chapters 4 and 5 were obtained from a single study. However, for the purpose of this dissertation the data are displayed in two chapters. Therefore, the two treatments and control groups were analyzed using ANOVA but in individual chapters data are shown between one monoclonal antibody treatment group and the isotype control group.

Introduction

The study of neutralizing monoclonal antibodies (mAbs) could be useful in the evaluation of the effectiveness of current Japanese encephalitis (JE) vaccines against emerging genotypes and strains. Specifically, the envelope (E) protein has been identified as a principal immunologic protein of Japanese encephalitis virus (JEV) and other related flaviviruses, making it a target of neutralizing antibody investigation (Lobigs and Diamond, 2012). Envelope protein domain III

(EDIII) mAbs have been described as having greater specificity and better neutralization potential than those recognizing other areas of the E protein, demonstrating protective efficacy when administered either prophylactically or therapeutically (Nybakken et al., 2005; Stettler et al., 2016).

Anti-Japanese encephalitis virus mAb JEV-31 targets EDIII and was selected to represent the protective antibody response against JEV. Several respective anti-EDIII mAbs isolated after immunization with JE vaccines have been described as strongly and broadly neutralizing among JEV genotypes (Calvert et al., 2019; Fan et al., 2015; Goncalvez et al., 2008). Similarly, JEV-31 was found to strongly neutralize genotypes I (GI), genotype II, genotype III (GIII), and genotype IV strains of JEV *in vitro* (genotype V was not evaluated) and provide protection in a murine model (Fernandez et al., 2018). As mAb JEV-31 is derived from a GIII JE vaccine strain, the current study seeks to evaluate efficacy against a GI strain to elucidate the protective ability that the GIII derived SA-14-14-2 vaccine would have against a strain of the emerging genotype. Unlike previous assessments, this investigation utilizes immune competent miniature swine to determine their utility in future assessments of JE vaccines.

Results

Serum neutralizing antibody titers

The immune protection threshold was exceeded in all the pigs that were passively immunized with mAb JEV-31, as shown in Figure 4-1 due to the presence of neutralizing antibodies. The serum neutralizing antibody titers were significantly higher in animals that received mAb JEV-31 than the isotype CHK-152 control pigs ($p < 0.05$). The isotype non-immune control group consisted of pigs receiving anti-chikungunya virus CHK-152 mAb. This was

utilized as the negative control as CHK-152 lacks specificity for JEV. The half-life of murine mAbs in pigs was determined to establish the efficacy of the passive transfer of murine mAb JEV-31 to miniature pigs in the time frame used in these experiments.

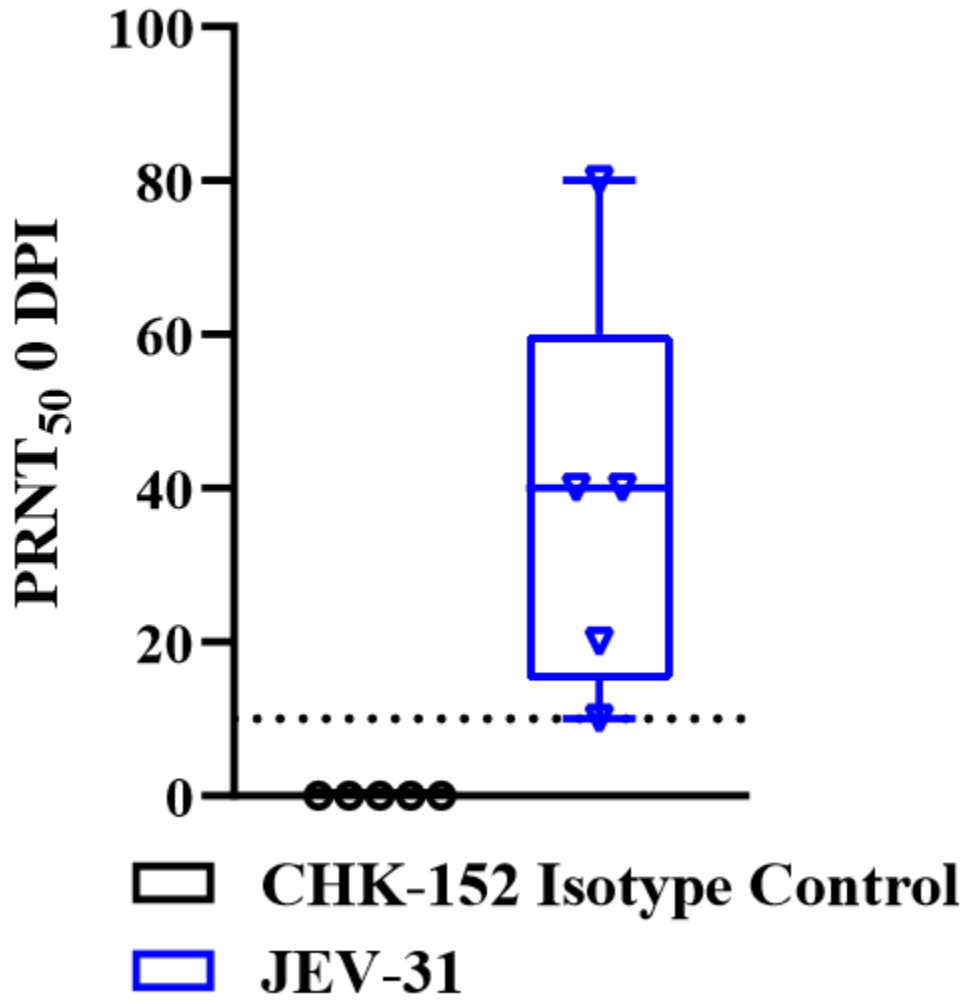

Figure 4-1. Individual $PRNT_{50}$ titers prior to challenge showing that the mAbs were not metabolized by the pigs. This demonstrated that the passive immunization of mAb JEV-31 effectively supplied neutralizing antibodies to miniature swine *in vivo*. There was a significant difference between groups receiving mAb CHK-152 and mAb JEV-31 ($p < 0.05$). The $PRNT_{50}$ titer data did not follow normal distribution. The data was analyzed using the Kruskal Wallis ANOVA followed by Dunnett's post hoc comparison. The box plot extends from the 25th to 75th percentiles. The line in the middle of the box is plotted at the median. The whiskers show the minimum and maximum values, and each individual data point is shown on the graph. The isotype

control group is represented in black with circles and JEV-31 is represented in blue with triangles. The dashed line at 10 represents the immune protection threshold against JEV (Hombach et al., 2005).

JEV-31 delays onset of fever

The presence, onset, and duration of fever was one of the disease signs assessed to determine the immune protection of mAb JEV-31. The pigs were monitored daily for clinical signs, with body temperatures taken over the course of the experiment. A body temperature greater or equal to 40°C was recognized as fever in the pigs, represented as a dashed line in Figure 4-2.

The CHK-152 group was found to have elevated body temperatures as early as one day post infection (DPI) with one (20%) animal having a fever (Figure 4-2). Over the duration of the study, this increased, with four (80%) pigs having a fever two DPI and all five (100%) pigs having a fever three DPI

The mAb JEV-31 effectively prevented the onset of fever as none of the pigs in the JEV-31 group had a fever up to two DPI. Only one of the five animals had an elevated body temperature at three DPI. The passive immunization of mAb JEV-31 significantly reduced the body temperature of pigs between two and three DPI when compared to the CHK-152 isotype control pigs ($p < 0.05$).

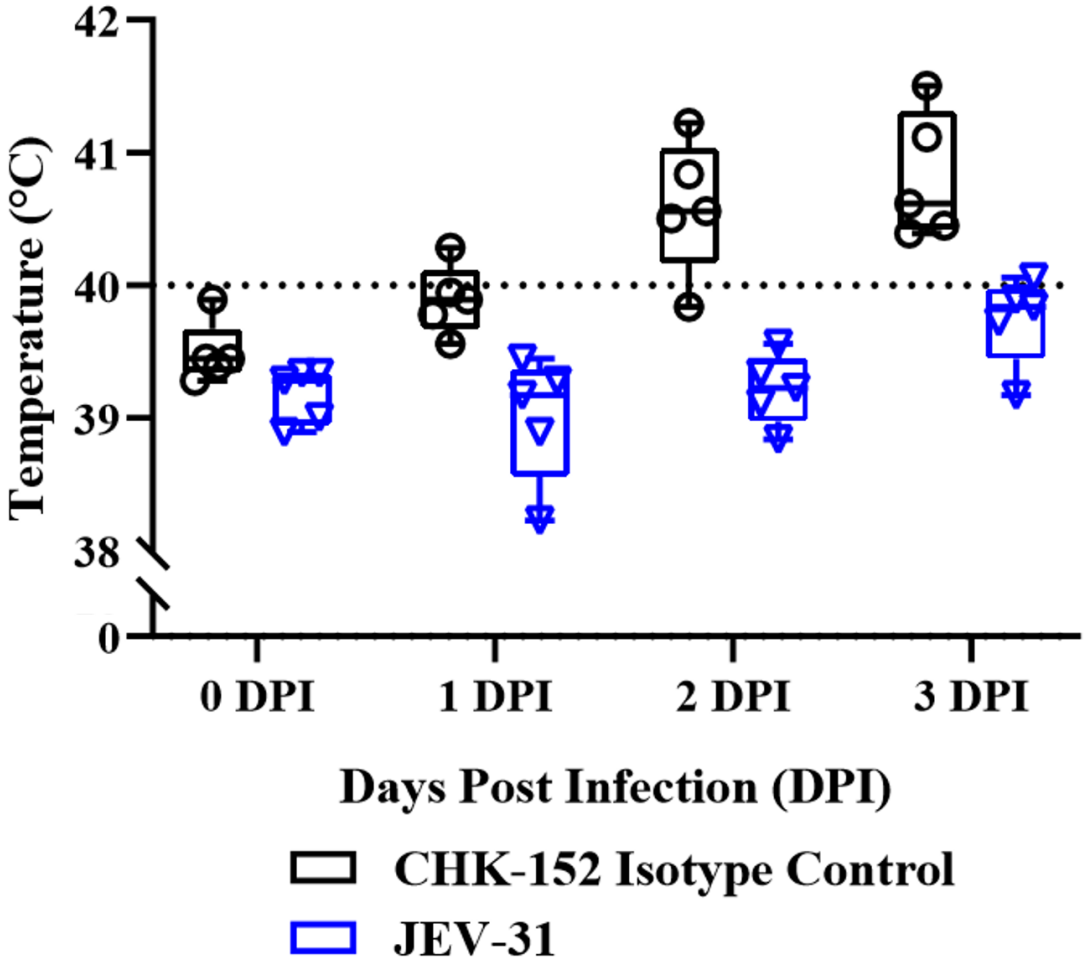

Figure 4-2. Individual temperatures with the dashed line representative of an elevated body temperature (> 40° Celsius). The passive immunization of mAb JEV-31 prevented the onset of fever in miniature swine until three days post infection when one pig developed a fever. The JEV-31 animals had a significantly lower body temperature two- and three-days post infection ($p < 0.05$). The temperature data did not follow normal distribution. The data was analyzed using the Kruskal Wallis ANOVA followed by Dunnett's post hoc comparison. The box plot extends from the 25th to 75th percentiles. The line in the middle of the box is plotted at the median. The whiskers show the minimum and maximum values, and each individual data point is shown on the graph. The isotype control group is represented in black with circles and JEV-31 is represented in blue with triangles.

JEV-31 decreased serum viral load

The serum viral load was compared between groups to determine the protective efficacy of JEV-31. Pigs that were passively transferred with the isotype control mAb CHK-152 developed transient viremia between one and two DPI, with detectable viral load in three (60%) pigs as early as one DPI as shown in Figure 4-3. The highest serum viral load occurred on two DPI in all five (100%) of the pigs with an average of 3.8 genome equivalents to $\log_{10}TCID_{50}$/mL.

Pigs passively immunized with mAb JEV-31 experienced a delayed onset of viremia, as well as a reduction in the highest observed serum viral load. In the JEV-31 group, the serum viral load at two DPI and three DPI was significantly reduced when compared to that of the isotype control group ($p < 0.05$). The highest observed viremic titer was significantly lower in the JEV-31 group, observed on three DPI when compared to the CHK-152 group peak titer on day two ($p < 0.05$).

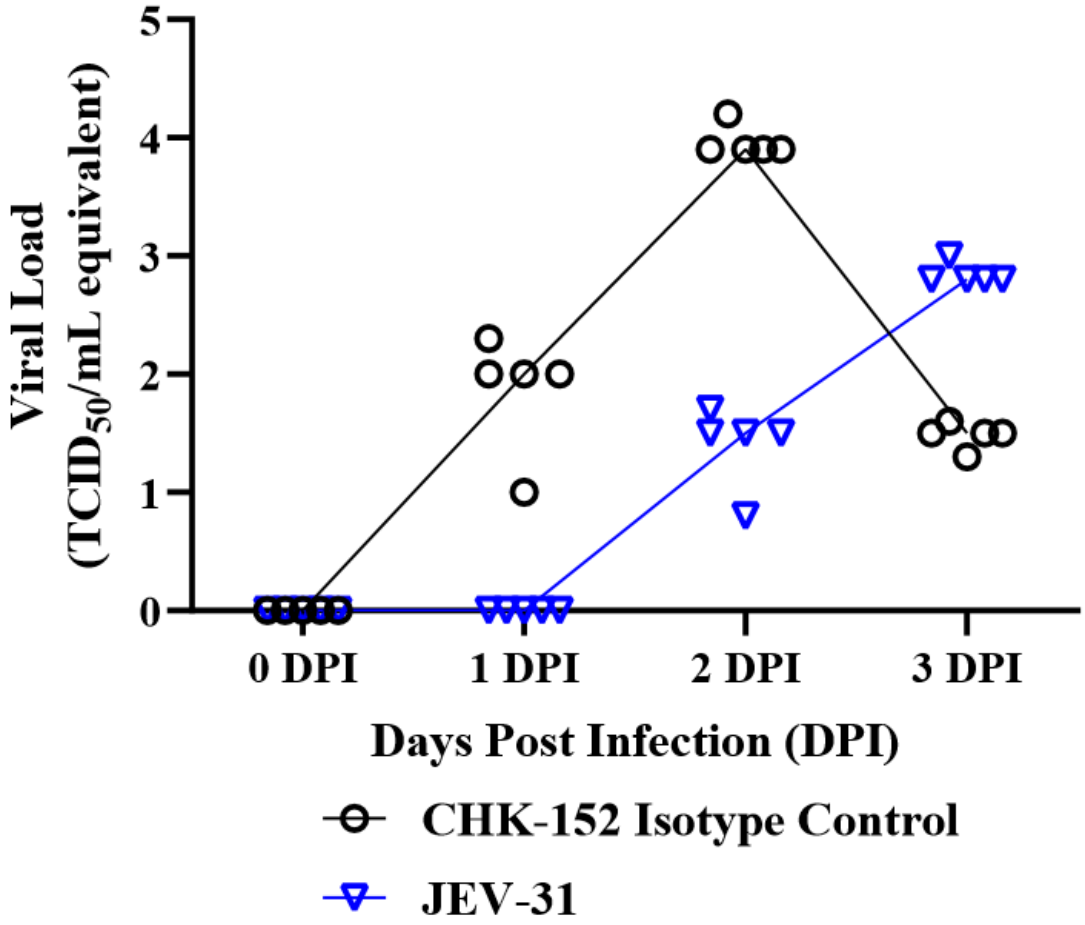

Figure 4-3. Viral titers of serum following JEV challenge in individual pigs. The pigs in the mAb JEV-31 group experienced a delayed onset of viremia, with the highest observed viral load on three days post infection. In contrast, pigs in the CHK-152 group developed viremia by one DPI reaching highest viral load on two DPI. Viral load in the serum was significantly lower in JEV-31 animals two- and three-days post infection ($p < 0.05$). The data did not follow normal distribution. The data was analyzed using the Kruskal Wallis ANOVA followed by Dunnett's post hoc comparison. The isotype control group is represented in black with circles and JEV-31 is represented in blue with triangles and plotted through the median.

JEV-31 reduces oral and nasal shedding

The oronasal shedding of JEV was evaluated in passively immunized pigs, given it is a pathological outcome observed in experimentally challenged swine (Lyons et al., 2018; Park et al., 2018; Ricklin et al., 2016a). Oral fluid was collected by suspending a twisted cotton rope 1.25 cm in diameter in each pen. Oronasal shedding in isotype control pigs began as early as one DPI increasing throughout the duration of the experiment (Figure 4-4). The pigs in the mAb JEV-31 group saw the lowest viral load in oral nasal shedding among the passively immunized animals (JEV-169 results are shown in chapter 5).

Control pigs began nasal shedding at one DPI with highest viral load observed on three DPI, which was after viremia had been cleared (Figure 4-5). Nasal shedding in pigs passively immunized with JEV-31 was significantly lower than the control group at three DPI ($p < 0.05$). This group also had a significant reduction in the viral load of the nasal epithelium compared to the isotype control CHK-152 pigs ($p < 0.05$) (Fig. 4-6).

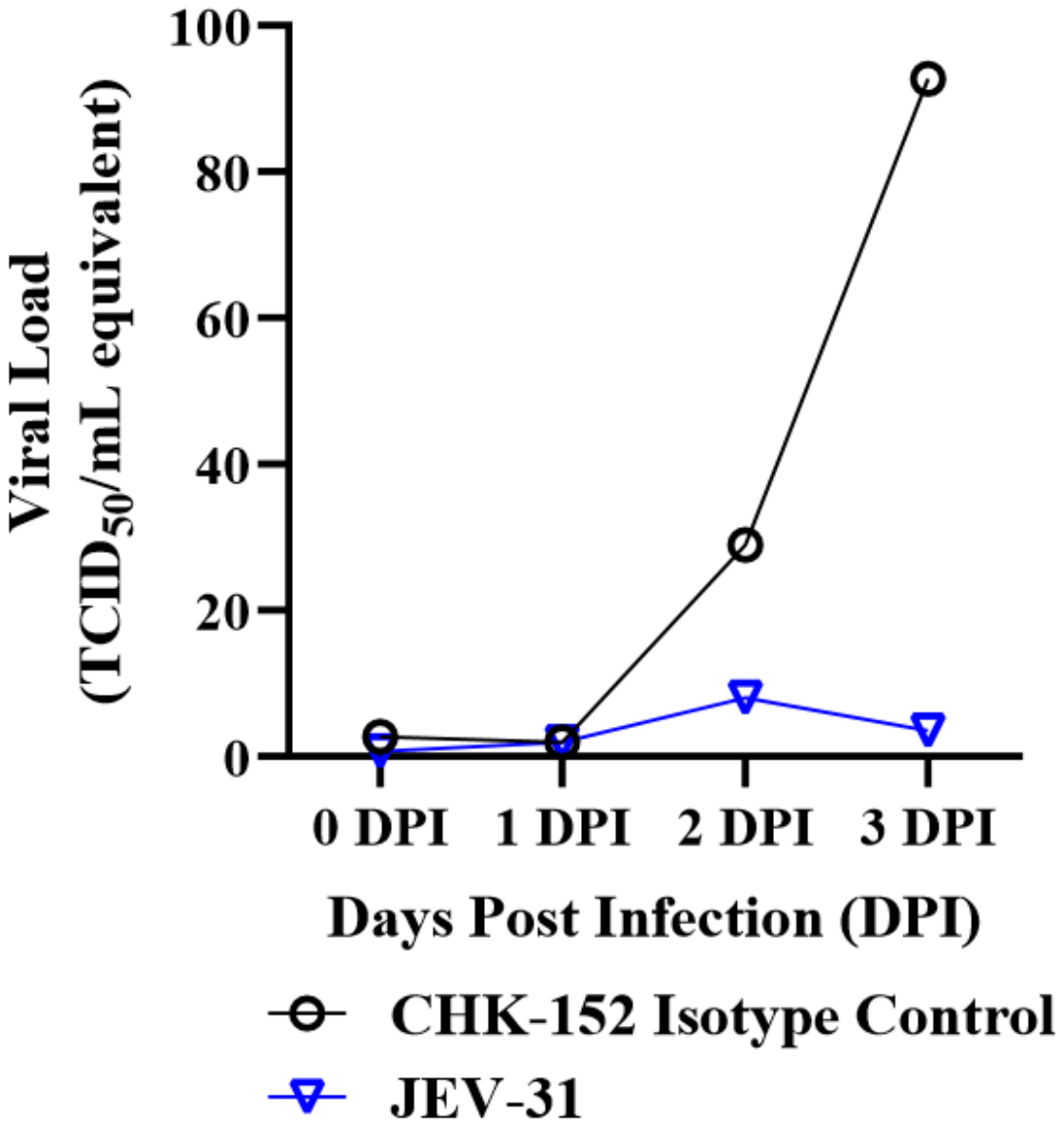

Figure 4-4. Viral load in oral fluid. Pen based oral fluid was collected daily with a cotton rope. The viral load in the oral fluid of the CHK-152 pigs increased during the experiment reaching highest observed levels on three days post infection. There was a reduction in the observed viral loads of pigs passively immunized with mAb JEV-31. The data did not follow normal distribution. Viral load in oral fluid data was analyzed using Kruskal Wallis ANOVA ($p > 0.05$). The isotype control group is represented in black with circles and JEV-31 is represented in blue with triangles.

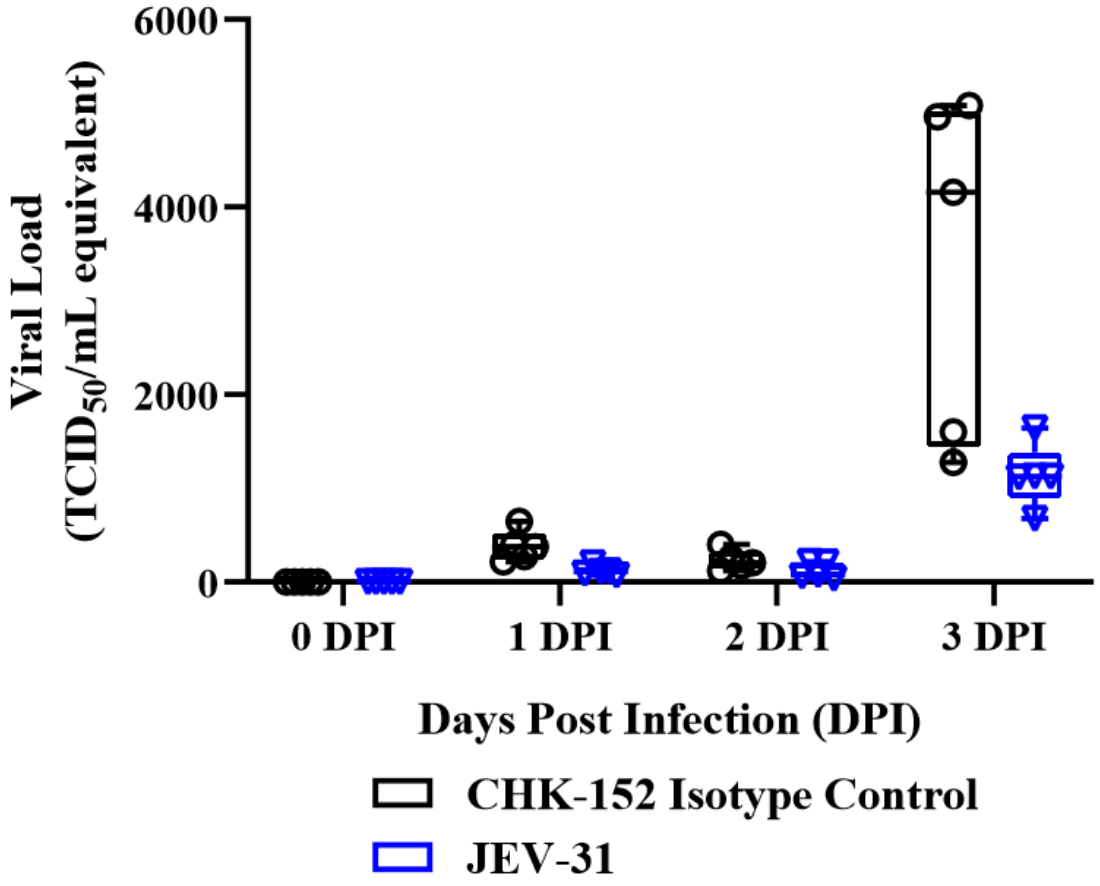

Figure 4-5. Viral load in nasal swabs. Nasal shedding in the CHK-152 pigs was observed by one day post infection reaching the highest observed viral load three days post infection. A reduction in viral load of the nasal swabs was seen throughout the experiment in the JEV-31 pigs, with a significant reduction in the viral load compared to the control at three days post infection ($p < 0.05$). The data did not follow normal distribution. The nasal shedding data was analyzed using the Kruskal Wallis ANOVA followed by Dunnett's post hoc comparison. The box plot extends from the 25th to 75th percentiles. The line in the middle of the box is plotted at the median. The whiskers show the minimum and maximum values, and each individual data point is shown on the graph. The isotype control group is represented in black with circles and JEV-31 is represented in blue with triangles.

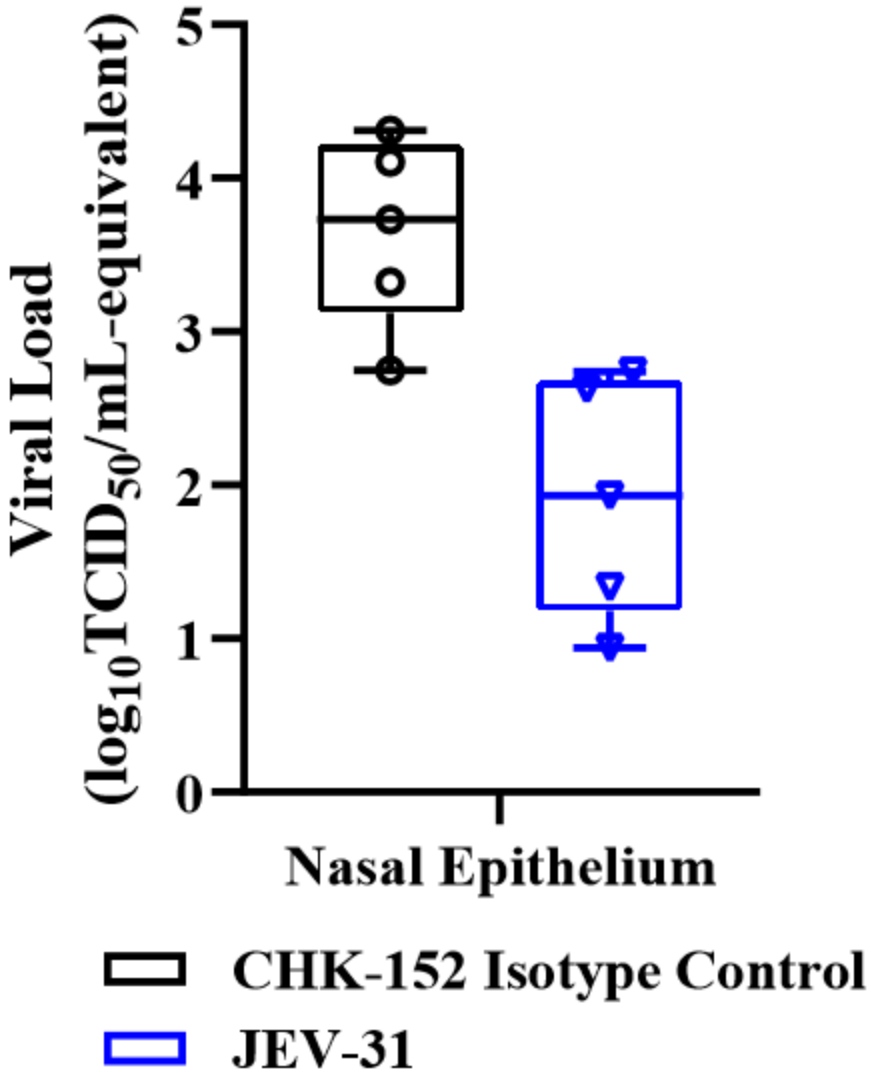

Figure 4-6. Viral load in nasal epithelium three DPI. Pigs passively immunized with JEV-31 had a significantly lower viral load in the nasal epithelium compared to the isotype controls ($p < 0.05$). Replication in the nasal epithelium could contribute to nasal shedding. The data did not follow normal distribution. The nasal epithelium data was analyzed using the Kruskal Wallis ANOVA followed by Dunnett's post hoc comparison. The box plot extends from the 25th to 75th percentiles. The line in the middle of the box is plotted at the median. The whiskers show the minimum and maximum values, and each individual data point is shown on the graph. The isotype control group is represented in black with circles and JEV-31 is represented in blue with triangles.

JEV-31 reduced fecal shedding

Fecal swabs were obtained from pigs three DPI to evaluate the shedding of virus through the digestive tract. While not statistically significant, pigs receiving mAb JEV-31 were observed to have experienced less viral shedding in fecal swabs than that of the isotope control CHK-152 group ($p > 0.05$) (Figure 4-7).

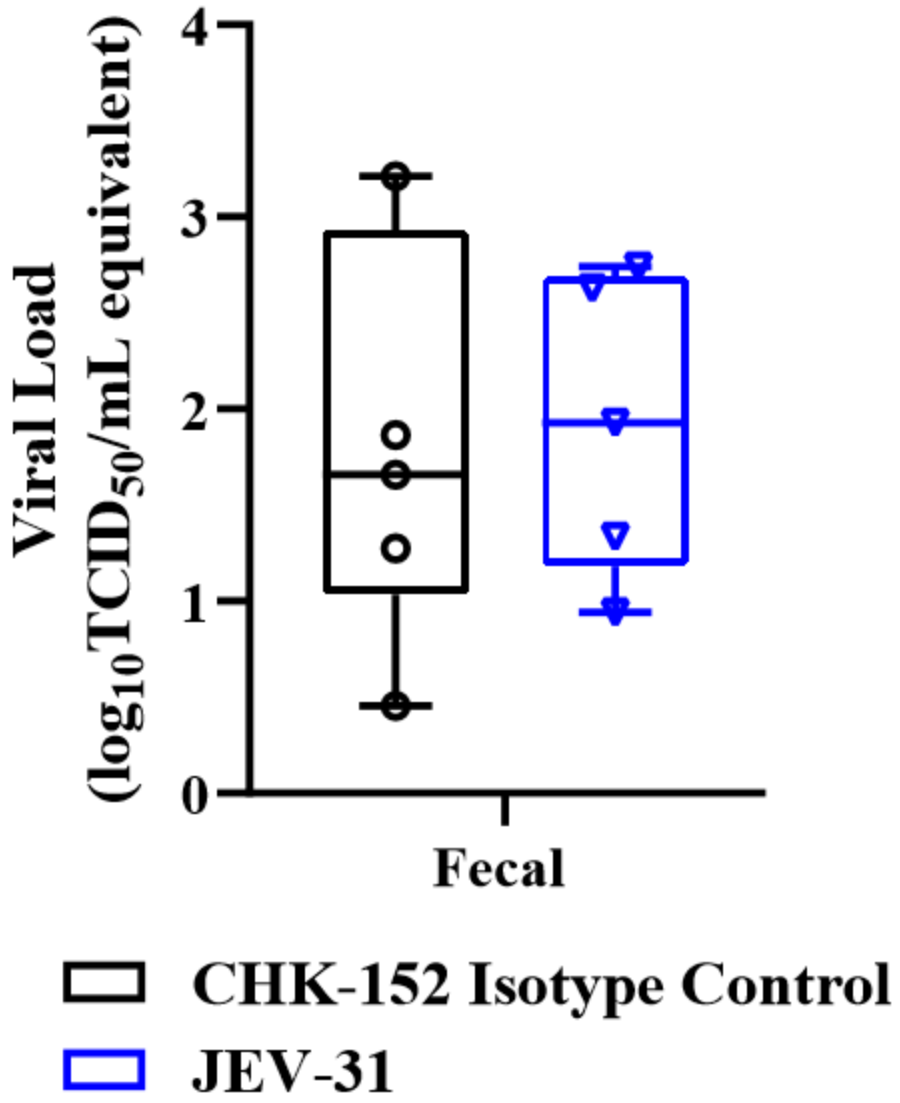

Figure 4-7. Viral load in fecal swabs three DPI. Fecal swabs were obtained from individual animals and demonstrated that pigs passively immunized with JEV-31 had a decreased viral load. This suggests decreased viral shedding through the digestive tract of JEV-31 pigs which could limit pig to pig transmission. The data did not follow normal distribution. The fecal shedding data was analyzed using the Kruskal Wallis ANOVA ($p > 0.05$). The box plot extends from the 25th to 75th percentiles. The line in the middle of the box is plotted at the median. The whiskers show the minimum and maximum values, and each individual data point is shown on the graph. The isotype control group is represented in black with circles and JEV-31 is represented in blue with triangles.

JEV-31 reduced lymphoid and central nervous tissue viral loads

Five central nervous tissues and six lymphoid tissues were quantified and evaluated to assess the protective efficacy of mAb JEV-31 against JEV neuroinvasion and systemic infection. In the isotype control mAb CHK-152 pigs, viremia was cleared at three DPI. However, positive detection of viral RNA was found in all the assessed tissues, showing that neuroinvasion and dissemination had occurred.

The reduction of JEV neuroinvasion was observed in the central nervous tissue of pigs receiving mAb JEV-31 (Figure 4-8). Pigs that received the passive transfer of mAb JEV-31 experienced significantly lower viral loads in central nervous tissues compared to that of the isotype control pigs ($p < 0.05$).

Of the six lymphoid tissues, all had a lower viral load in pigs that were passively transferred with mAb JEV-31 ($p < 0.05$) (Figure 4-9), with all but the mesenteric lymph node having a significant reduction in the viral load. The average viral load of the lymphoid tissues of pigs in the CHK-152 group exceeded 3.5 $\log_{10}TCID_{50}$/mL genome equivalent.

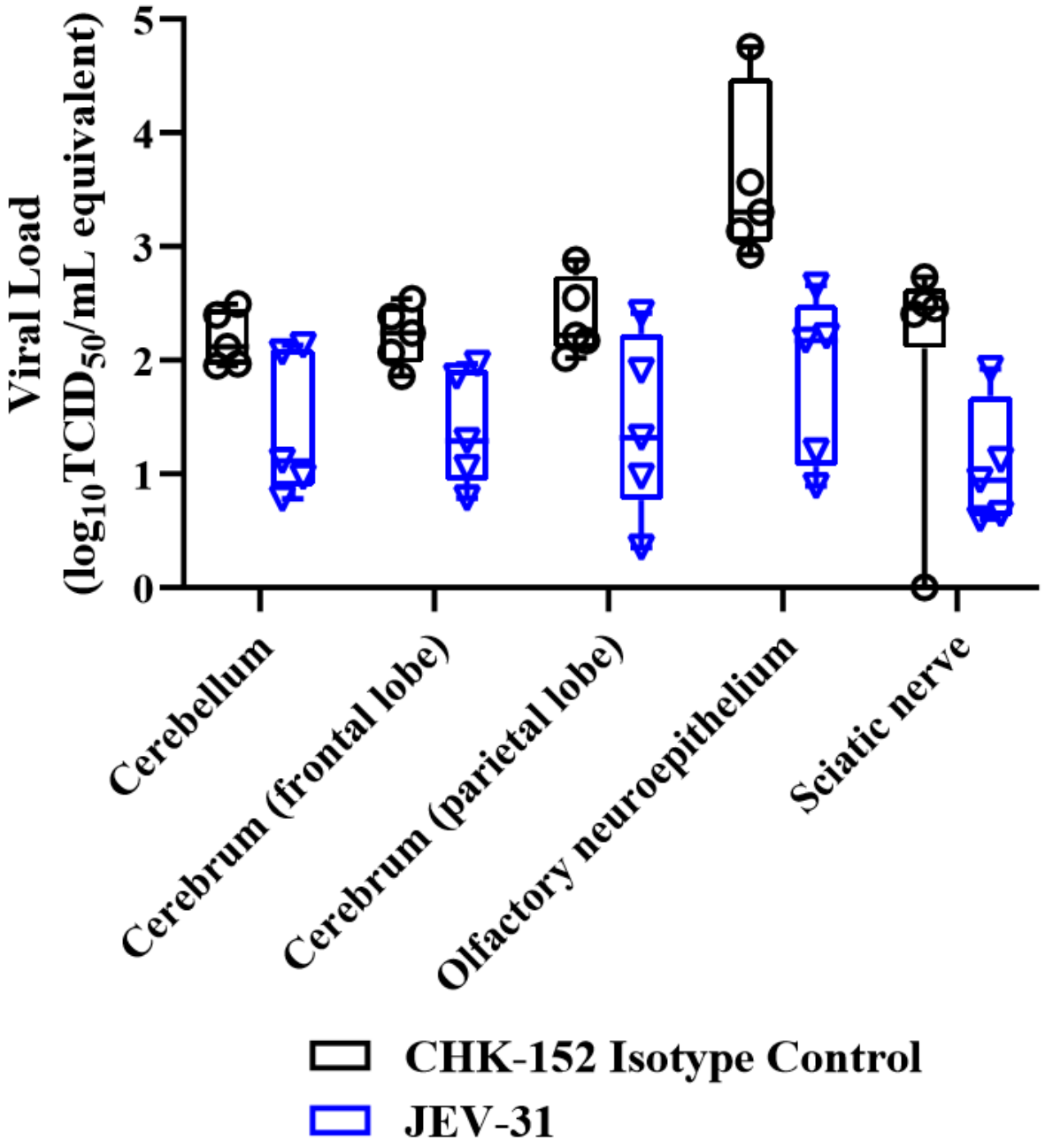

Figure 4-8. Viral load in brain tissue three DPI. Pigs that received JEV-31 had a significant reduction in the five tissues of the central nervous system compared to the isotype control pigs. The tissue data besides the cerebellum did not follow normal distribution. The tissue data except the cerebellum was analyzed using the Kruskal Wallis ANOVA and post hoc Dunnett's test ($p < 0.05$). The cerebellum data was normally distributed and was analyzed with one-way ANOVA and post hoc Dunnett's test ($p < 0.05$). The box plot extends from the 25th to 75th percentiles. The line in the middle of the box is plotted at the median. The whiskers show the minimum and

maximum values, and each individual data point is shown on the graph. The isotype control group is represented in black with circles and JEV-31 is represented in blue with triangles.

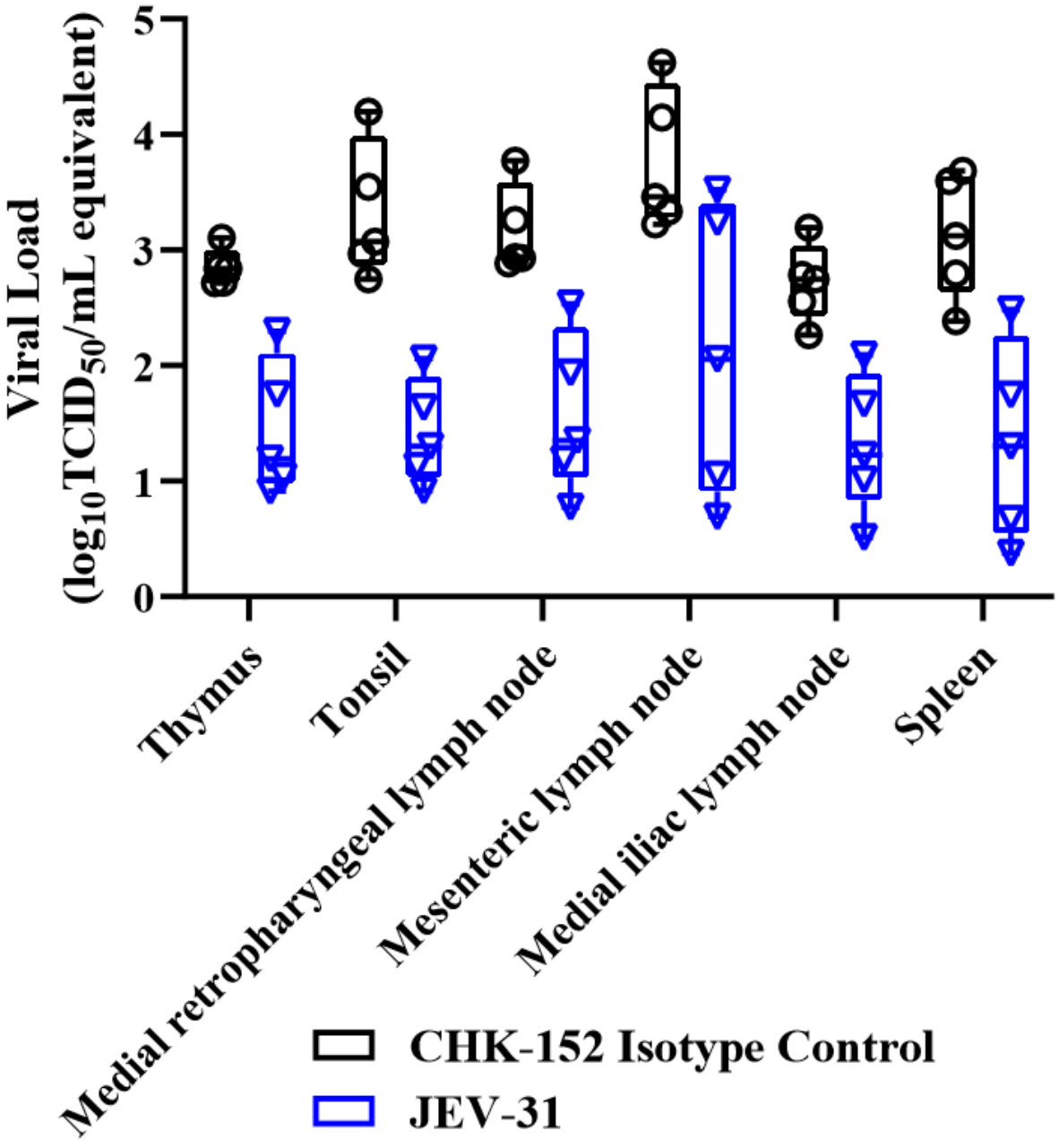

Figure 4-9. Viral load in lymphoid tissue three DPI. The passive transfer of JEV-31 reduced the viral load of six lymphoid tissues. There was a significant reduction observed in the thymus, tonsil, medial retropharyngeal lymph node, medial iliac lymph node, and spleen ($p < 0.05$). There was not a significant reduction in the mesenteric lymph node ($p > 0.05$). The tissue data did not follow normal distribution. The tissue data was analyzed using the Kruskal Wallis ANOVA and

post hoc Dunnett's test ($p < 0.05$). The box plot extends from the 25th to 75th percentiles. The line in the middle of the box is plotted at the median. The whiskers show the minimum and maximum values, and each individual data point is shown on the graph. The isotype control group is represented in black with circles and JEV-31 is represented in blue with triangles.

Discussion

This investigation of mAb JEV-31 aimed to determine if neutralizing antibodies against the EDIII of JEV could be of interest in the success of JE vaccines, providing protection against a strain of the emerging genotype. It was determined that mAb JEV-31 provided protection *in vivo* in miniature swine, suggesting future use of miniature swine to study JEV *in vivo*. The mAb JEV-31 is cross-reactive with JE serocomplex member WNV and has a binding profile similar to other complex-reactive flavivirus antibodies, responsive to residues within the EDIII lateral ridge region (Fig. 4-10).

In this study pigs passively immunized with mAb JEV-31 had serum titers that exceeded the threshold of protection against natural infection with greater than 10 $PRNT_{50}$ titers, demonstrating contributions of mAb JEV-31 against JEV infection (Hombach et al., 2005). This protection corresponded to a reduction in febrile illness, viremia, oronasal shedding, fecal shedding, and tissue viral load. These reductions were observed despite pigs in the JEV-31group having a lower $PRNT_{50}$ than pigs in the JEV-169 group, as will be shown in the next chapter. Furthermore, pigs passively immunized with mAb JEV-31 had a delay in the onset of viremia and reduction in the serum viral load.

These results suggest that the epitope recognized by mAb JEV-31 could be of importance in JE vaccines against emerging GI strains. The mAb JEV-31 is against the EDIII lateral ridge and other antibodies recognizing this region have demonstrated protection from different genotypes and cross reactivity with serocomplex members. This includes mAb 17BD3-2, which

similar to JEV-31 recognizes G333 (Calvert et al., 2019). The mAb 17BD3-2 was derived from a GIII vaccine and neutralized strains of GI, GII, and GIII *in vitro* (Calvert et al., 2019). Epitopes within EDIII have been determined to be important in the binding of JEV serocomplex cross-reactive mAbs, including G332, which is recognized by mAb JEV-31 (Chiou et al., 2012). A mouse derived mAb, 9F12, against dengue virus (DENV) serotype two is cross-reactive with the other three DENV serotypes and West Nile virus (Rajamanonmani et al., 2009). The 9F12 mAb recognizes an epitope in the EDIII lateral ridge similar to mAb JEV-31 (Rajamanonmani et al., 2009). Antibodies such as these against the EDIII lateral ridge are not as prevalent in the human response as EDI-EDII antibodies, though they have demonstrated the ability to neutralize multiple serotypes of DENV (Beltramello et al., 2010). The decrease in viral load observed in miniature pigs passively immunized with anti-EDIII mAb JEV-31 suggest that antibodies against this region significantly contribute to protection.

Another major takeaway from this work is the possibility of miniature swine for use in JE vaccine investigations. The pigs in this work were challenged intradermally with JEV to mimic natural infection and the isotype control pigs experienced disease symptoms similar to human infection. Additionally, it demonstrated that a mouse anti-JEV IgG antibody was able to protect the pigs from systemic JE infection and neurotropic disease, suggesting that miniature swine could be used to characterize antibody mediated protection against encephalitic flaviviruses.

Figure 4-10. Residues that constitute the epitope recognized by murine derived monoclonal antibody JEV-31 in the crystal structure of the JEV E protein (PDB access number: 3P54). The residues recognized by JEV-31 are discontinuous and located throughout the EDIII lateral ridge region and are depicted as purple spheres. Domains I, II, and III of the E protein are colored in red, yellow, and blue respectively. Epitope mapping was conducted by Fernandez et al. using both hydrogen-deuterium exchange mass spectrometry and alanine-scanning site-directed mutagenesis (Fernandez et al., 2018).

Chapter 5 - Protective Efficacy of Vaccination-Derived Anti-Japanese Encephalitis Virus Monoclonal Antibody JEV-169 in Miniature Swine

This chapter is adapted from a manuscript published in Antiviral Research:

Young, C. L., Lyons, A. C., Hsu, W.-W., Vanlandingham, D. L., Park, S. L., Bilyeu, A. N., Ayers, V.B., Hettenbach, S.M., Zelenka, A.M., Cool, K.C., Peterson, G.J., Higgs, H., Huang, Y.-J. S. (2020). Protection of swine by potent neutralizing anti-Japanese encephalitis virus monoclonal antibodies derived from vaccination. Antiviral Research, 174, 104675. doi: https://doi.org/10.1016/j.antiviral.2019.104675

* Data displayed in chapters 4 and 5 were obtained from a single study. However, for the purpose of this dissertation the data are displayed in two chapters. Therefore, the two treatments and control groups were analyzed using ANOVA but in individual chapters data are shown between one monoclonal antibody treatment group and the isotype control group.

Introduction

Potently neutralizing mAbs against the envelope (E) protein can contribute to the immune protection generated by Japanese encephalitis (JE) vaccines. As it has previously been shown that mAbs reactive with an interdomain epitope, an epitope between domains, are important for protection against flavivirus infections and vaccines, mAb JEV-169 that recognizes a discontinuous epitope throughout envelope protein domain I (EDI) and envelope protein domain

II (EDII) was selected to evaluate protection in miniature swine (Fibriansah et al., 2014; Goncalvez et al., 2008). Neutralizing mAbs that recognize similar regions to mAb JEV-169 have been described against dengue virus (DENV) and yellow fever virus (YFV) demonstrating immune protection (Lai et al., 2007; Men et al., 2004; Ryman et al., 1998). The same immunocompetent miniature swine used to assess mAb JEV-31 in chapter 4 was utilized for this evaluation of genotype III (GIII) vaccine derived mAb JEV-169 to provide further insight on the ability of current JE vaccines to protect against emerging genotype I (GI) strains. This further allowed assessment of miniature swine as a platform to evaluate the protective efficacy of neutralizing mAbs against JEV *in vivo*.

Results

Serum neutralizing antibody titers

The control group for this experiment consisted of isotype non-immune pigs that received anti-chikungunya virus CHK-152 mAb. This was utilized because it allowed for antibody characteristics to be matched without the specificity for the target antigen (JEV). The serum of pigs passively immunized with JEV-169 was significantly more neutralizing than the isotype control CHK-152 ($p < 0.05$), exceeding the immune protection threshold which is considered the immunological correlate for protection against JE (Figure 5-1). The neutralization titers were determined using serum collected at challenge, to demonstrate that the passive transfer of murine mAb JEV-169 was an effective approach for delivery in the pigs.

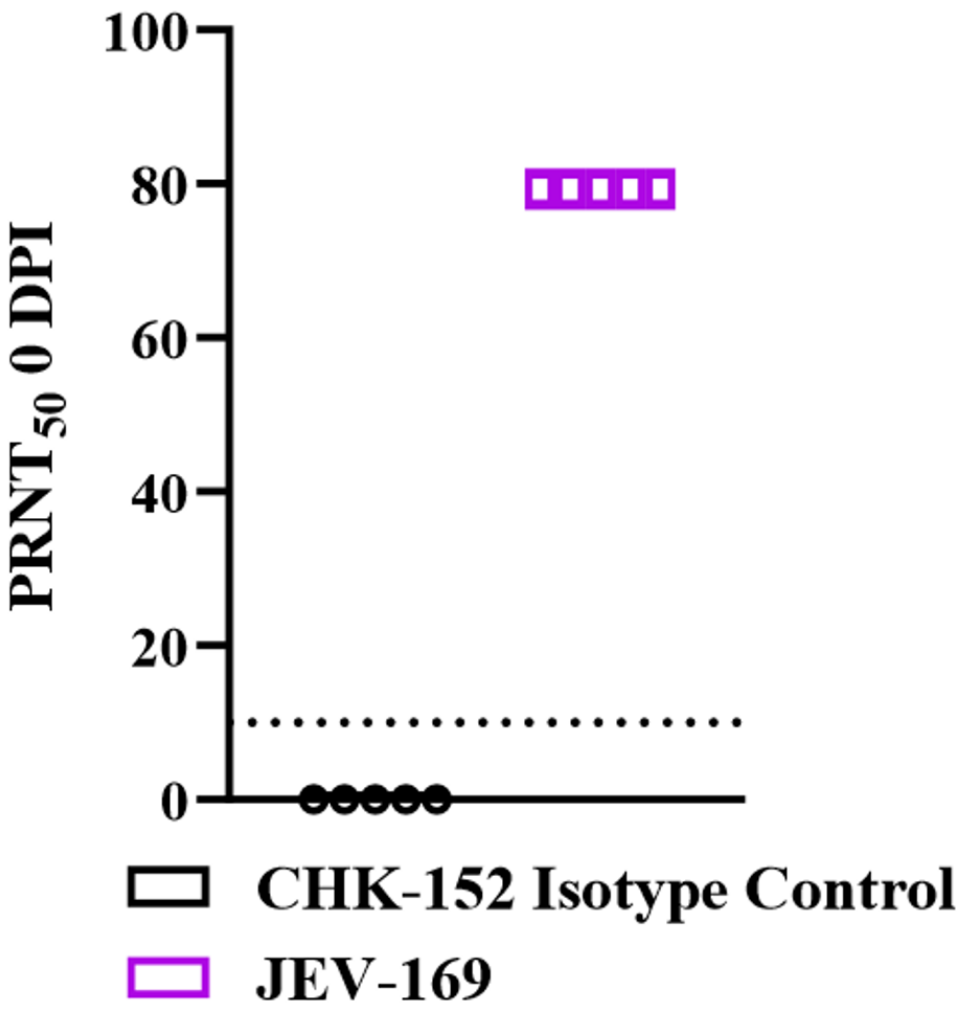

Figure 5-1. Individual $PRNT_{50}$ titers prior to challenge showing that the mAbs were not metabolized by the pigs. There was a significant difference between groups receiving mAb CHK-152 and mAb JEV-169 ($p < 0.05$). The $PRNT_{50}$ titer data did not follow normal distribution. The data was analyzed using the Kruskal Wallis ANOVA followed by Dunnett's post hoc comparison. The isotype control group is represented in black with circles and JEV-169 is represented in purple with squares. The dashed line at 10 represents the immune protection threshold against JEV (Hombach et al., 2005).

JEV-169 prevented fever

The passive transfer of mAb JEV-169 was able to effectively prevent fever in all five pigs for the duration of the experiment (Figure 5-2). Taken daily, body temperatures greater or equal to 40°C were recognized as a fever. None of the pigs in the JEV-169 group had a fever or elevated temperature. When compared to the isotype control group animals, the JEV-169 group had significantly lower body temperatures two and three DPI ($p < 0.05$). Lack of fever and healthy presentation of these pigs indicated significant protection against JEV challenge.

The isotype control pigs had pigs with elevated body temperatures as early as one day post infection (DPI), with one pig (20%) with a temperature above 40°C. By two DPI there were four pigs (80%) with an elevated body temperature, and all the pigs in the CHK-152 group had a fever on three DPI

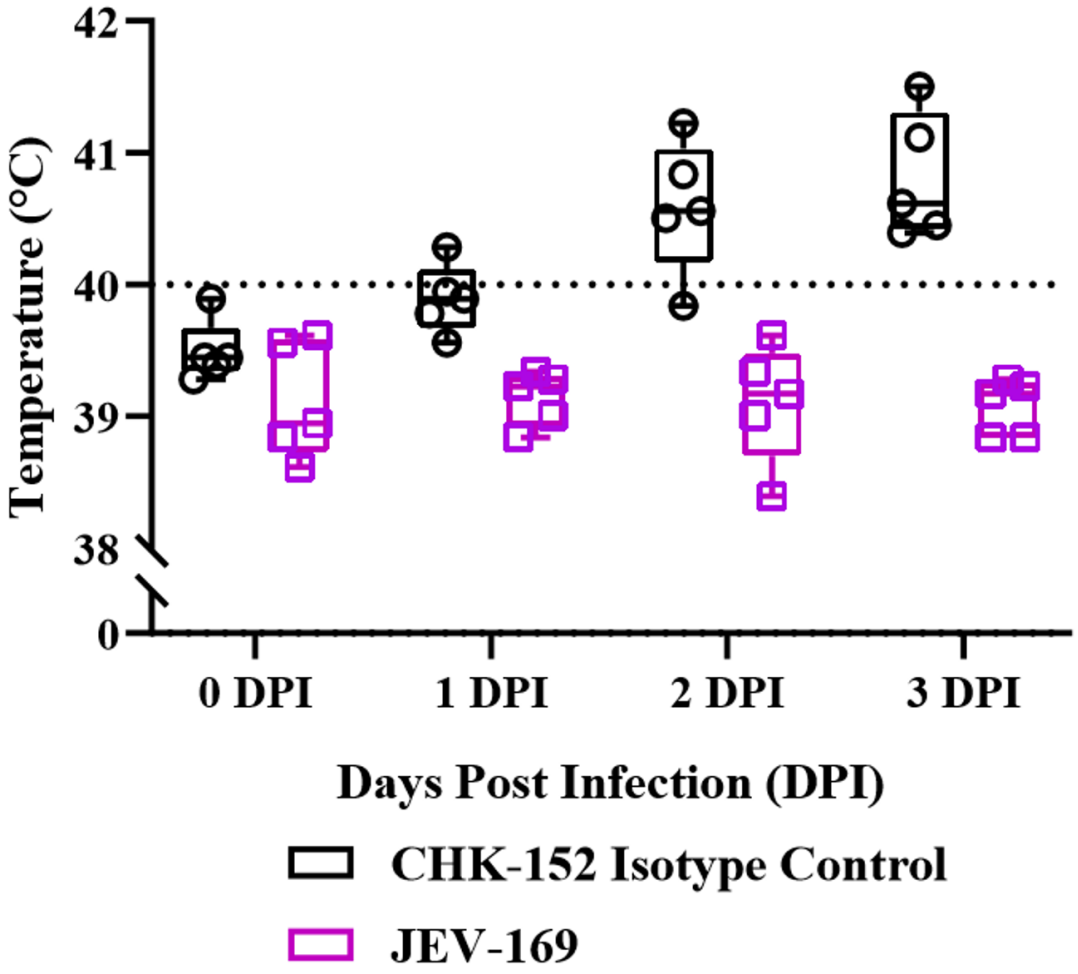

Figure 5-2. Individual temperatures with the dashed line representative of an elevated body temperature (> 40° Celsius). In the isotype control group, pigs began to experience a fever as early as one day post infection with all animals in this group having an elevated body temperature three days post infection. In contrast, no pigs passively immunized with JEV-169 had a fever during the experiment. Temperature was significantly lower in JEV-169 animals two- and three-days post infection ($p < 0.05$). The temperature data did not follow normal distribution. The data was analyzed using the Kruskal Wallis ANOVA followed by Dunnett's post hoc comparison. The box plot extends from the 25th to 75th percentiles. The line in the middle of the box is plotted at the median. The whiskers show the minimum and maximum values, and each individual data point is shown on the graph. The isotype control group is represented in black with circles and JEV-169 is represented in purple with squares.

JEV-169 reduces viral load in serum

The serum viral load at two and three DPI was significantly lower than that of the isotype control group ($p < 0.05$) in the mAb JEV-169 pigs (Figure 5-3). The pigs that were passively immunized with mAb JEV-169 experienced peak viremia on two DPI, with a significant reduction in the serum viral load compared to the peak viremia of the isotype control CHK-152 ($p < 0.05$).

Pigs that were passively transferred with isotype control mAb CHK-152 developed viremia between one and two DPI. There was detectable viral load in the serum of three pigs as early as one DPI with the highest serum viral load of the CHK-152 pigs occurring on two DPI.

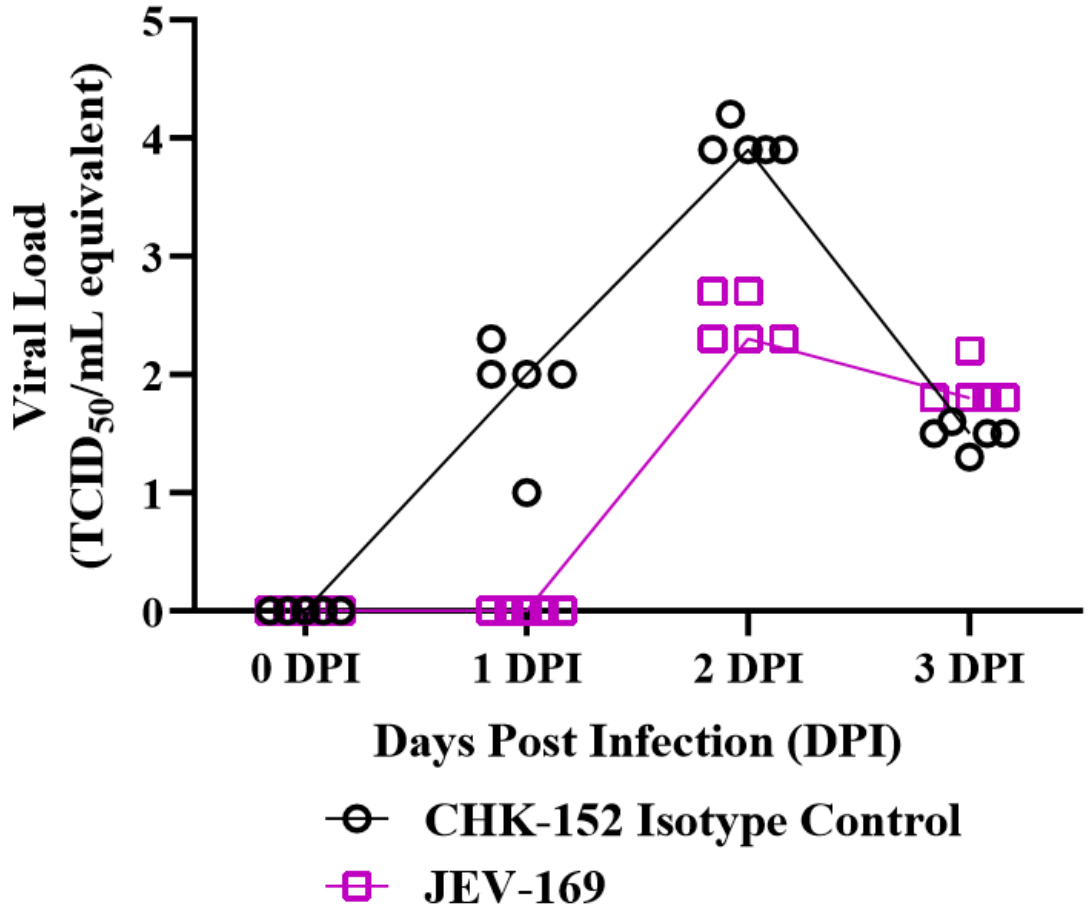

Figure 5-3. Viral titers of serum following JEV challenge in individual pigs. Viremia was observed in pigs in the isotype control group by one day post infection, and peak viremia was seen on two days post infection. In the pigs passively immunized with JEV-169 peak viremia was observed on two days post infection with a significant reduction in the viral load when compared to the isotype controls ($p > 0.05$). The data did not follow normal distribution. The data was analyzed using the Kruskal Wallis ANOVA followed by Dunnett's post hoc comparison. The isotype control group is represented in black with circles and JEV-169 is represented in purple with squares and plotted through the median.

JEV-169 reduces oral and nasal shedding of JEV

The shedding of JEV in oral fluid in isotype control pigs began as early as one DPI and increased throughout the duration of the experiment. The increase in oral fluid shedding was observed in the absence of viremia (Figure 5-4). Pigs in the JEV-169 group experienced lower viral load in oral fluids than that of the isotype control CHK-152 pigs, demonstrating the ability of JEV-169 to reduce shedding of JEV in oral fluids.

The nasal shedding of control animals began one DPI with the highest viral load detected on three DPI after viremia had been cleared (Figure 5-5). The nasal shedding of the pigs that were passively immunized with mAb JEV-169 was significantly lower at three DPI, compared to the control group. Pigs passively immunized with JEV-169 also saw significantly reduced viral loads in the nasal epithelium ($p < 0.05$) (Figure 5-6).

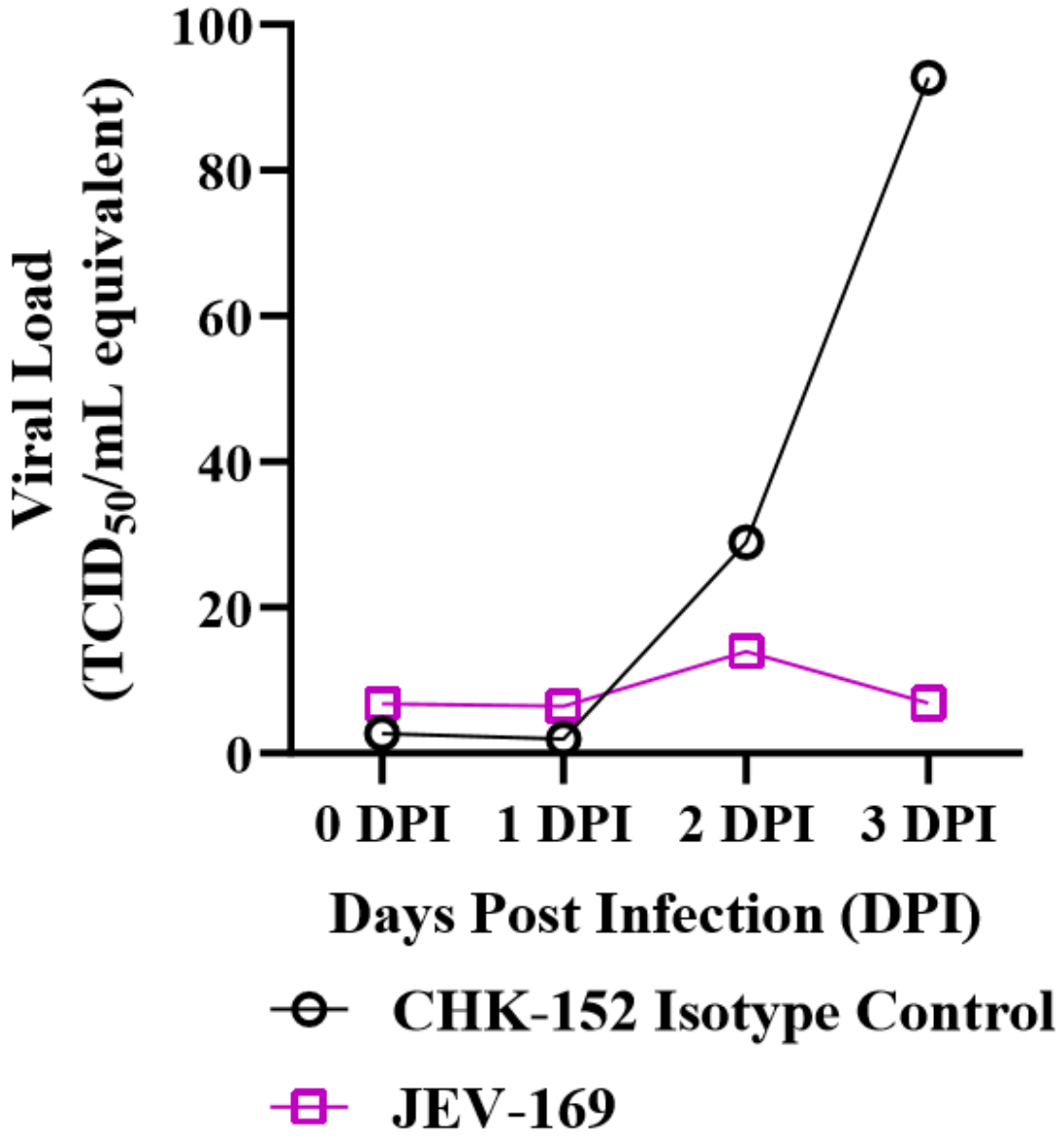

Figure 5-4. Viral load in oral fluid. Pen based oral fluid was collected daily. Shedding in oral fluid of the isotype control pigs increased throughout the experiment. Pigs in the JEV-169 group experienced a reduction in the viral loads of oral fluid. The data did not follow normal distribution. Viral load in oral fluid data was analyzed using Kruskal Wallis ANOVA ($p > 0.05$). The isotype control group is represented in black with circles and JEV-169 is represented in purple with squares.

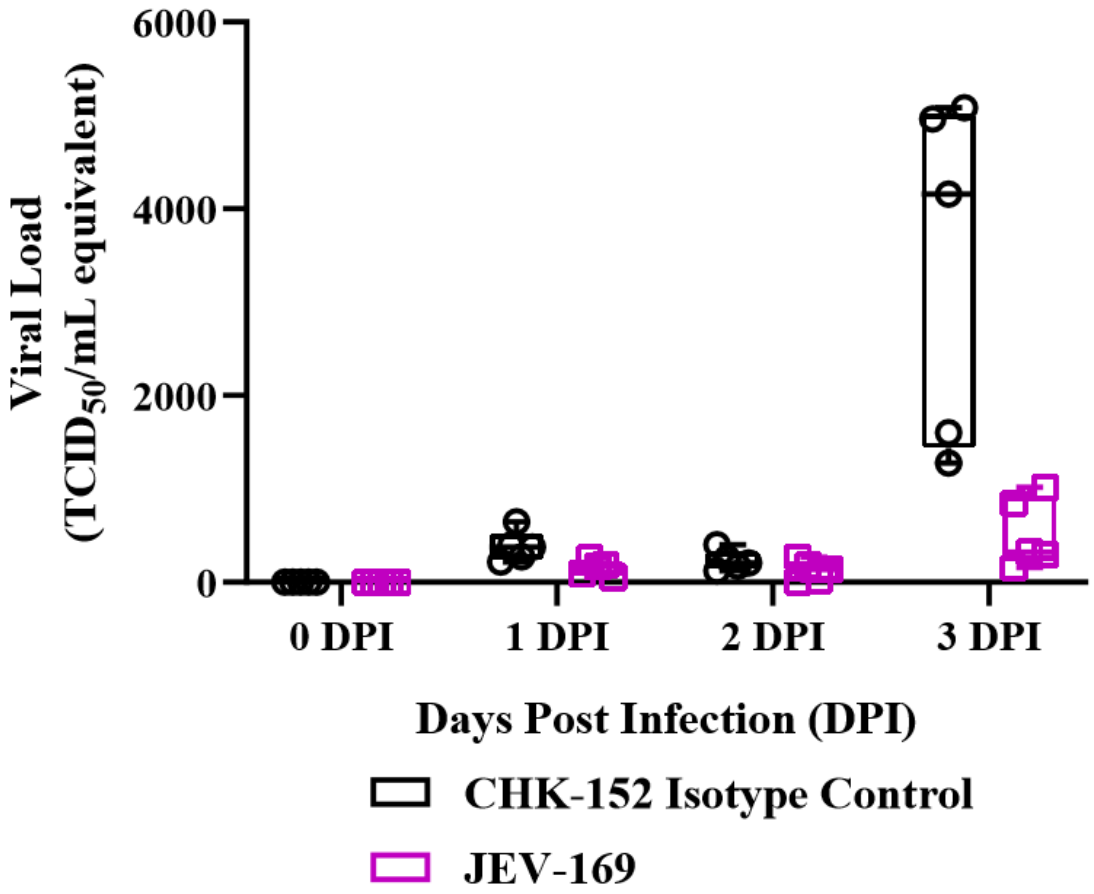

Figure 5-5. Viral load in nasal swabs. Viral load in the nasal swabs of isotype control pigs reached its highest observed level on three days post infection. The viral load of nasal swabs on the JEV-169 pigs was significantly less than the control pigs on three days post infection ($p < 0.05$). The data did not follow normal distribution. The nasal shedding data was analyzed using the Kruskal Wallis ANOVA followed by Dunnett's post hoc comparison. The box plot extends from the 25th to 75th percentiles. The line in the middle of the box is plotted at the median. The whiskers show the minimum and maximum values, and each individual data point is shown on the graph. The isotype control group is represented in black with circles and JEV-169 is represented in purple with squares.

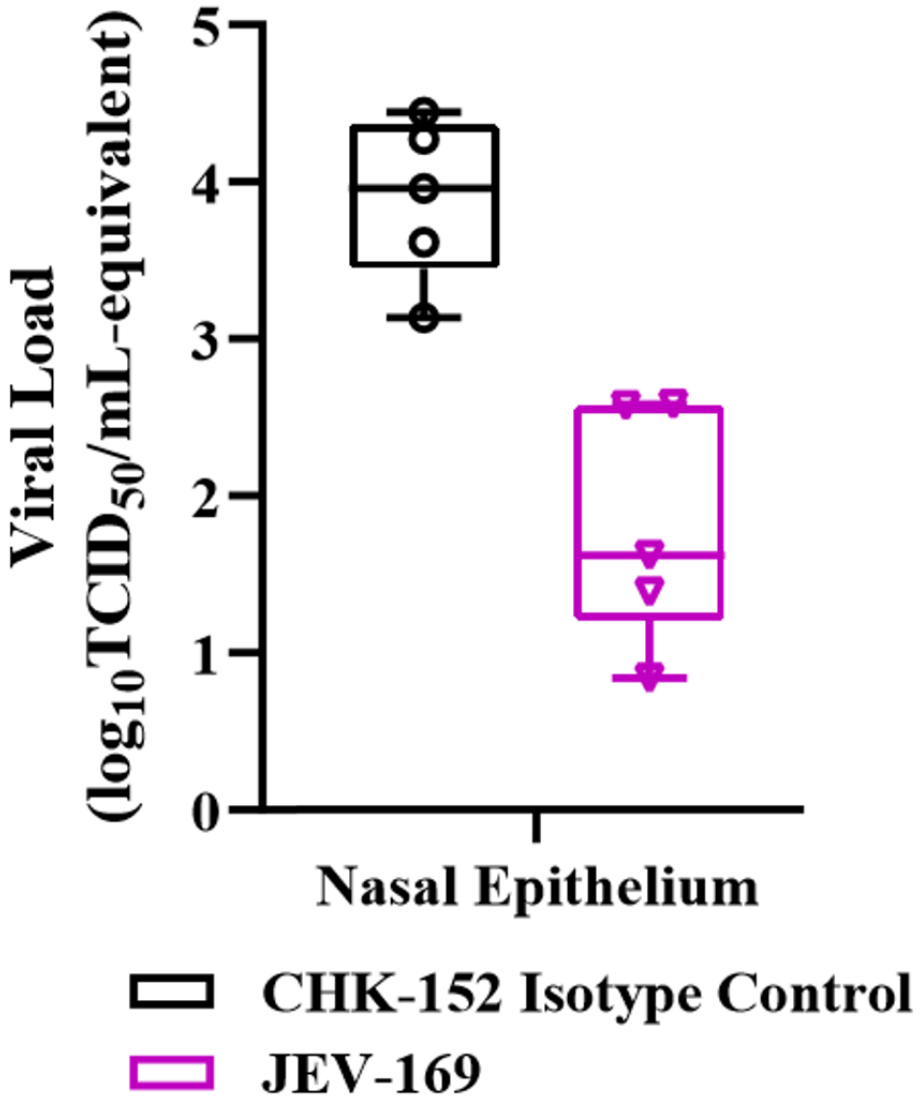

Figure 5-6. Viral load in nasal epithelium three DPI. Pigs passively immunized with JEV-169 had a significantly lower viral load in the nasal epithelium compared to the isotype controls ($p < 0.05$). Replication in the nasal epithelium could contribute to nasal shedding. The data did not follow normal distribution. The nasal epithelium data was analyzed using the Kruskal Wallis ANOVA followed by Dunnett's post hoc comparison. The box plot extends from the 25th to 75th percentiles. The line in the middle of the box is plotted at the median. The whiskers show the minimum and maximum values, and each individual data point is shown on the graph. The isotype control group is represented in black with circles and JEV-169 is represented in purple with squares.

JEV-169 reduced fecal shedding

Pigs receiving mAb JEV-169 had less viral shedding observed in fecal swabs than that of the isotope control CHK-152 group, though it was not statistically significant ($p > 0.05$) (Figure 5-7). Fecal swabs were obtained from pigs three DPI to evaluate the shedding of virus through the digestive tract.

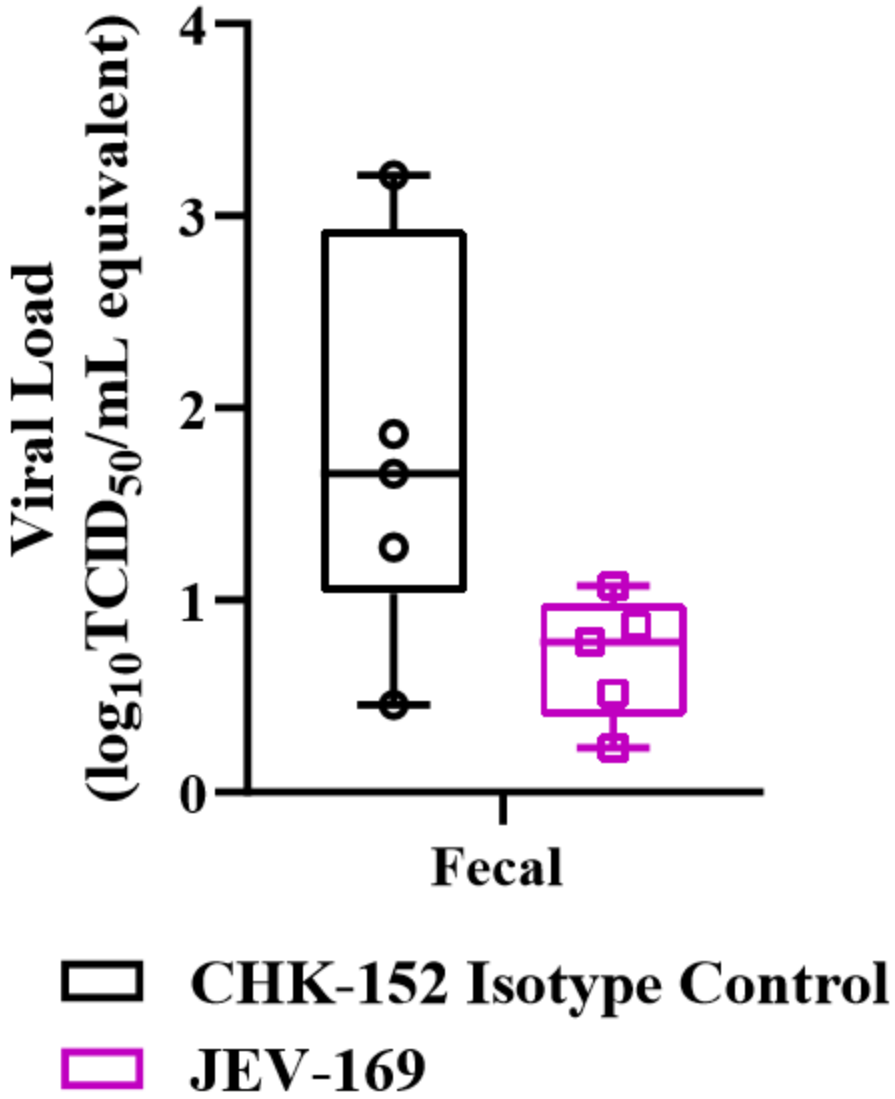

Figure 5-7. Viral load in fecal swab at three DPI. Fecal swabs were obtained from individual animals and demonstrated that pigs passively immunized with JEV-169 had a decreased viral load. This suggests decreased viral shedding through the digestive tract of JEV-31 pigs which could limit pig to pig transmission. The data did not follow normal distribution. The fecal shedding data was analyzed using the Kruskal Wallis ANOVA ($p > 0.05$). The box plot extends from the 25th to 75th percentiles. The line in the middle of the box is plotted at the median. The whiskers show the minimum and maximum values, and each individual data point is shown on the graph. The isotype control group is represented in black with circles and JEV-169 is represented in purple with squares.

JEV-169 reduced lymphoid and central nervous tissue viral loads

The reduction of JEV neuroinvasion was observed in the central nervous tissues of pigs that received mAb JEV-169 (Figure 5-8). Pigs that received the passive transfer of mAb JEV-169 had significantly lower viral loads in the central nervous tissues that were evaluated than the isotype control pigs ($p < 0.05$). Of the six lymphoid tissues, all had a significantly lower viral load in pigs in the mAb JEV-169 group when compared to controls with the exception of the mesenteric lymph node ($p < 0.05$) (Figure 5-9).

Pigs receiving the isotype control mAb CHK-152 had detectable viral loads in all primary and secondary lymphoid tissues exceeding that of the JEV-169 group. While the isotype control mAb CHK-152 pigs viremia cleared at three DPI, positive detection of viral RNA was found in all the tissues evaluated. This suggests that neuroinvasion and dissemination in the lymphoid tissue in the CHK-152 animals occurred.

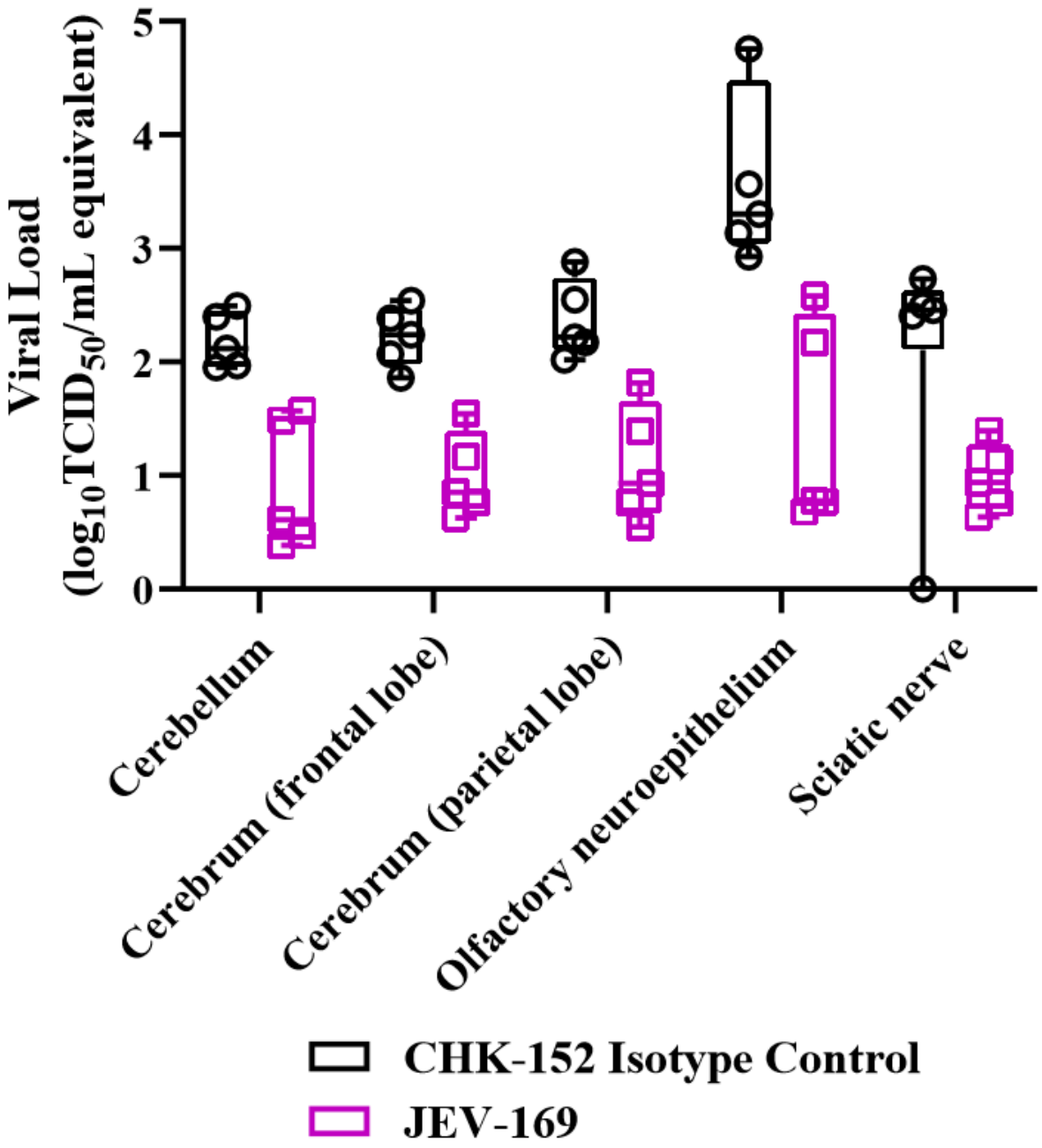

Figure 5-8. Viral load in brain tissue. Pigs that received JEV-169 had a significant reduction in the five tissues of the central nervous system compared to the isotype control pigs ($p < 0.05$). The tissue data besides the cerebellum did not follow normal distribution. The tissue data except the cerebellum was analyzed using the Kruskal Wallis ANOVA and post hoc Dunnett's test ($p < 0.05$). The cerebellum data was normally distributed and was analyzed with one-way ANOVA and post hoc Dunnett's test ($p < 0.05$). The box plot extends from the 25th to 75th percentiles. The line in the middle of the box is plotted at the median. The whiskers show the minimum and maximum

values, and each individual data point is shown on the graph. The isotype control group is represented in black with circles and JEV-169 is represented in purple with squares.

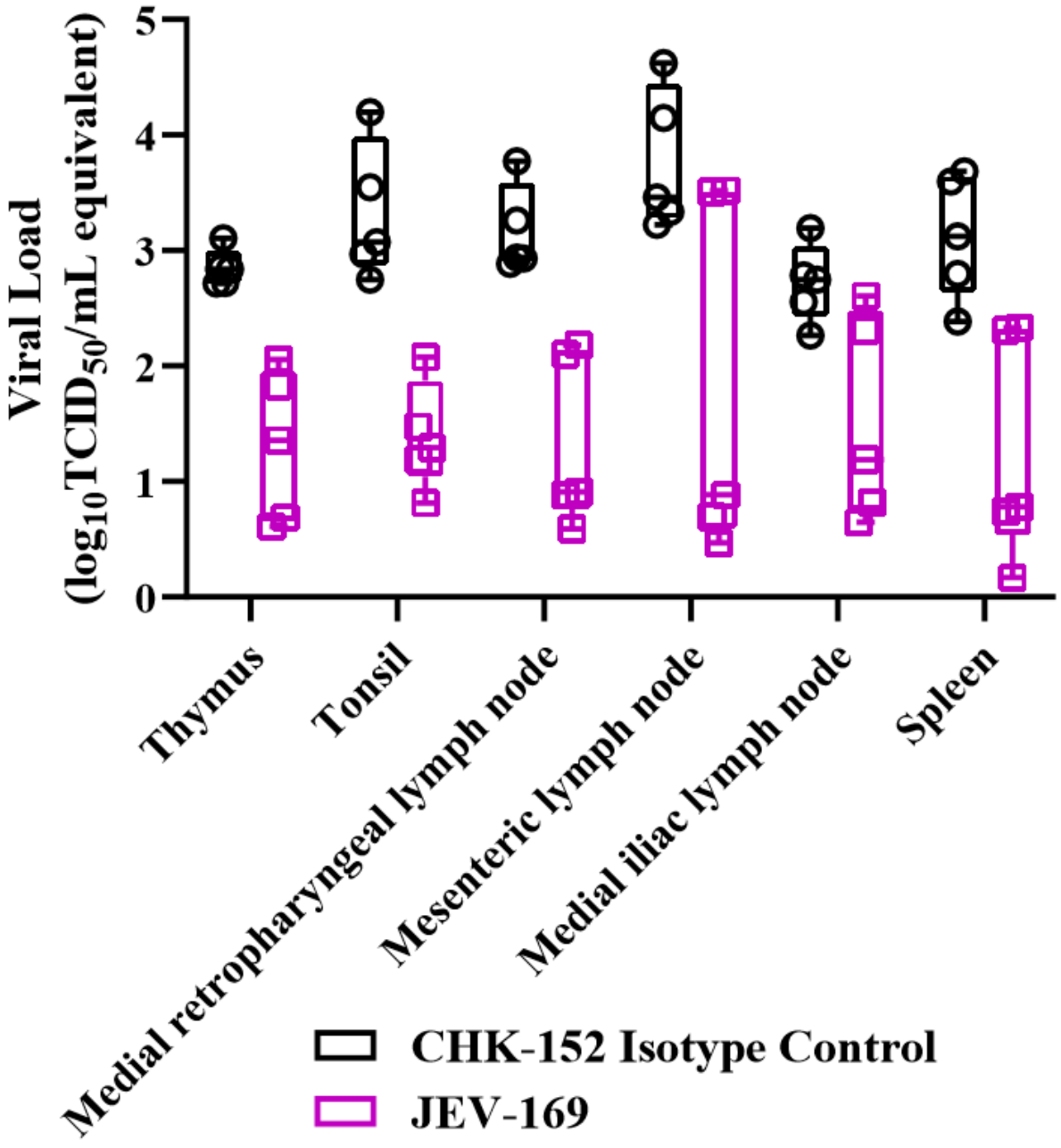

Figure 5-9. Viral load in lymphoid tissue. The passive transfer of JEV-169 reduced the viral load of six lymphoid tissues. There was a significant reduction observed in the thymus, tonsil, medial retropharyngeal lymph node, medial iliac lymph node, and spleen of JEV-169 pigs ($p < 0.05$). There was not a significant reduction in the viral load of the mesenteric lymph node ($p >$

0.05). There was not a significant reduction in the mesenteric lymph node ($p > 0.05$). The tissue data did not follow normal distribution. The tissue data was analyzed using the Kruskal Wallis ANOVA and post hoc Dunnett's test ($p < 0.05$). The box plot extends from the 25^{th} to 75^{th} percentiles. The line in the middle of the box is plotted at the median. The whiskers show the minimum and maximum values, and each individual data point is shown on the graph. The isotype control group is represented in black with circles and JEV-31 is represented in blue with triangles.

Discussion

Similar to mAb JEV-31, the passive immunization of mAb JEV-169 exceeded the threshold of protection against infection with greater than 10 $PRNT_{50}$ titers in the serum of miniature swine. The pigs that were passively immunized with JEV-169 had the highest $PRNT_{50}$ compared to the other experimental groups. Another important finding was that pigs passively immunized with JEV-169 had improved outcomes in regard to multiple JE clinical signs. None of the pigs in this group experienced elevated body temperatures and saw reductions in oronasal shedding and tissue viral loads.

Monoclonal antibodies against EDI-EDII have demonstrated protection against DENV (Fibriansah et al., 2014; Goncalvez et al., 2008). The human mAb 1F4 was able to neutralize DENV serotype one *in vitro* and in a mouse model (Fibriansah et al., 2014). A chimpanzee derived anti-EDI-EDII mAb, B2, has previously been demonstrated to be highly neutralizing against multiple strains of JEV and was the most protective against *in vivo* challenge in mice (Goncalvez et al., 2008).

The epitope recognized by mAb JEV-169 spans EDI and EDII and could be important in current vaccines, inducing an immune response against GI strains (Fig. 5-10). Similar regions are recognized in the anti-DENV serotype four mAb 5H2 (Lai et al., 2007). This anti-EDI mAb provided protection *in vivo* against DENV serotype four challenge (Lai et al., 2007). The anti-

YFV mAb 117 also recognizes an epitope in the EDI region (Ryman et al., 1998). The mAb 117 is specific for the Asibi wild type YFV strain (Gould et al., 1989).

Insight was also provided into how current JE vaccination strategies could be optimized using miniature swine. Intradermal experimental challenge with GI JE-91 induced pathological outcomes that resembled human JE infection. Future studies of interdomain epitopes could be performed to determine protection against the JEV GV Muar strain, as an ideal vaccine candidate designed for JE would be able to broadly protect against multiple genotypes and strains.

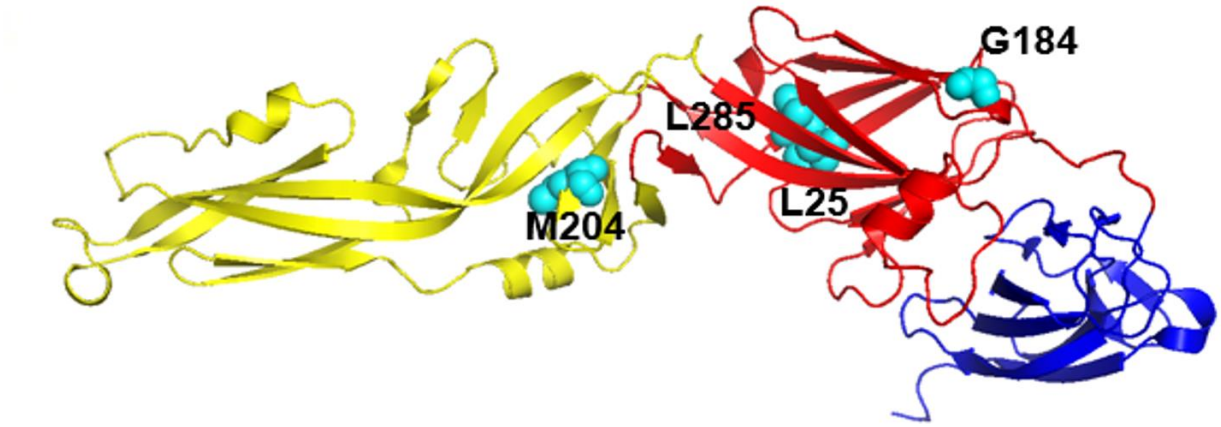

Figure 5-10. Residues that constitute the epitope recognized by murine derived monoclonal antibody JEV-169 in the crystal structure of the JEV E protein (PDB access number: 3P54). The residues recognized by JEV-31 are located in the EDI/EDII regions and depicted as cyan spheres. Domains I, II, and III of the E protein are colored in red, yellow, and blue respectively. Epitope mapping was conducted by Fernandez et al. using both hydrogen-deuterium exchange mass spectrometry and alanine-scanning site-directed mutagenesis (Fernandez et al., 2018).

Chapter 6 - Conclusion, Discussion, and Future Direction

The work presented in this dissertation provides information to potentially develop countermeasures against emerging flaviviruses. In this chapter, the major conclusions of this work are summarized, and future directions to continue to improve flavivirus prevention and control measures are provided.

Vector competence of North American mosquitoes to flaviviruses

Determining vector competence is important to assess whether North American mosquitoes are capable of transmitting flaviviruses that have not yet been detected in the continent. Given the impact of global change on the emergence of infectious diseases, it is paramount that North American vectors are assessed for their potential to transmit arboviruses to create informed mitigation procedures in advance of, or in the event of, an outbreak.

While the emergence of several mosquito-borne flaviviruses to the New World is plausible, Usutu (USUV) was chosen for this evaluation in North American mosquitoes. The USUV was selected for this study due to its recent emergence across Europe. As seen with the introduction of West Nile virus (WNV), inter-continental introductions of arboviruses do occur, and with increased human mobility, may become more common in future years. As WNV is widely distributed across the globe infecting similar mosquitoes and vertebrate hosts, Japanese encephalitis (JE) serocomplex member USUV is of major public health importance to North America.

Two North American *Culex* species mosquitoes were competent vectors for USUV based on acquired infection, disseminated infection, and USUV detection in the saliva demonstrating transmission potential. This suggests USUV was able to initiate infection in the midgut,

disseminate into the secondary tissues, and infect the salivary glands. North American *Culex* mosquitoes have also been determined to by susceptible to Japanese encephalitis virus (JEV), suggesting that targeting these species for surveillance and control would be beneficial to improve the efficiency of flavivirus prevention programs (Huang et al., 2015).

Since USUV is an emerging flavivirus further studies will be required to understand which factors are supporting its emergence and how biological and environmental aspects interact and could affect its activity and spread. In addition, the knowledge of other potential mosquito vectors and vertebrate hosts of USUV will provide information that can be used to improve flavivirus control measures.

Miniature swine for evaluation of Japanese encephalitis virus

An important objective of this dissertation was the determination of the suitability and usefulness of miniature swine for future assessments of JE vaccines and therapeutics. Miniature swine could be used for investigations of JE vaccine efficacy as they would be a relevant *in vivo* platform for JEV infection studies. The results included within this dissertation support the feasibility of swine in JE vaccine assessment studies, which could be useful in the event of an outbreak of JE to a naïve population.

Utilization of swine would benefit JE vaccine studies, because as a natural host swine provide the advantage of demonstrating interactions between the host and pathogen using the applicable biological context (Kiros et al., 2012). This could help develop control measures, including discovering efficient management methods in the host that could then disrupt the transmission cycle. The biological information gained from using natural disease models has led

to large animal models being employed for vaccine design and testing of other infectious diseases such as Rift Valley fever and WNV (Kiros et al., 2012; Kroeker et al., 2020).

Swine are relevant to study JE as they are amplifying hosts that develop viremia and help sustain the transmission cycle of JEV in nature. The utilization of outbred immunocompetent swine to study JE is unique, given that many mAbs have been studied in murine models, which can be inbred and/or immunocompromised, or non-human primates, which generate ethical concerns and high research costs (Marín-Lopez et al., 2019). Swine provide the opportunity to study JE in an amplifying host while also allowing for the investigation of immune protection in an outbred and immunocompetent animal.

Prior to the work in this dissertation, the anti-JEV mAbs JEV-31 and JEV-169 had been assessed in a C57BL/6 mouse model. A popular inbred strain in research, C57BL/6 mice are genetically identical with uniform characteristics and traits. This means that some of the benefits of swine, such as an outbred response and individuality, are lacking. Diversity within swine could offer benefits to vaccine testing, facilitating determinations of safety and efficacy (Meurens et al., 2012).

When C57BL/6 mice were used to assess if anti-JEV mAbs could provide protection against JEV induced lethality, percent survival was the sole evaluated outcome (Fernandez et al., 2018). While mice have been utilized as models for JEV infection, different strains can vary in disease severity, not always consistently leading to outcomes related to human disease (Wang and Deubel, 2011). Swine are more closely aligned with human infection with regard to clinical presentations such as the development of fever and viremia. Similar responses to infection means that collected tissue and body fluid samples could be further aligned with what would be expected outcomes of human disease (Walters and Prather, 2013).

In this study, miniature swine were utilized to assess the *in vivo* protection of anti-JEV mAbs when administered prophylactically. Previous work demonstrated that miniature swine are highly susceptible to JE infection developing neurotropic disease (Park et al., 2018). Given that pathological outcomes can be induced through the experimental challenge of miniature swine, protection induced by the prophylactic passive immunization of anti-JEV mAbs was assessed. In these experiments, mAb JEV-31 and JEV-169 were found to be protective in miniature swine through the reduction of disease symptoms and viral load in tissues.

As similar pathogenesis can be observed in both human and swine infected with JEV, miniature swine could be a relevant *in vivo* platform. The evidence found in these experiments suggest that the mAbs could provide similar protection in humans as was witnessed in swine. Miniature swine offer an immunocompetent animal to study JEV infection and pathogenesis that can be used to evaluate correlates of protection. This could support the further use of swine in the development of JE vaccines and therapeutics, as well as highlight the usefulness of the miniature swine in the future design of prevention measures against other swine zoonotic diseases.

Passive transfer of anti-JEV mAbs reduces viremia, neuroinvasion, and tissue viral load

The clearance of viremia in pigs generally takes place within a few days (Scherer et al., 1959). Following the trend of natural infection, pigs in this experiment experienced peak viremia between two- and three-days post infection (DPI). The passive transfer of mAb JEV-31, however, delayed the onset of viremia and reduced the viral titer. Pigs that received the passive transfer of mAb JEV-169 experienced peak viremia at two DPI, similar to the isotype control group; however, the viral load was lower than that of the isotype control group as well as the JEV-31 group.

Given that JEV can cause severe and permanent neurological complications, it was important to evaluate the mAbs and assess their potential ability to prevent the neuroinvasion of JEV. The assessed tissues of the central nervous system were the cerebellum, cerebrum (frontal and parietal lobes), olfactory neuroepithelium, and the sciatic nerve. All five central nervous tissues showed a reduction in the viral loads of the pigs that were immunized with the anti-JEV mAbs.

Viral RNA was assessed in both primary and secondary lymphoid tissues including the thymus, tonsil, medial retropharyngeal lymph node, mesenteric lymph node, medial iliac lymph node, and spleen. Pigs receiving either of the anti-JEV mAbs had reduced tissue viral loads when compared to animals in the isotype control group. The reduction of the viral load of JEV through the lymphoid tissue emphasizes the protective potential of the anti-JEV mAbs and could suggest improved prognoses with their use.

JEV detection in oral fluids, nasal swabs, and fecal swabs

The utilization of oral fluid to diagnose swine pathogens has increased over recent years with the improvement and development of novel assays and reagents. Oral fluid can be obtained quickly through non-invasive rope-based procedures that do not require skilled personnel. Rope-based collection methods limit the amount of people needed for collection as the animals do not have to be individually restrained making it possible to simultaneously sample multiple animals in a pen, thereby decreasing costs and allowing testing of a large number of animals.

Results from these experiments demonstrated that the viral shedding of JEV in oral fluids can occur as early as two DPI, but that the passive transfer of either mAb JEV-31 or JEV-169 could reduce the viral load. The evidence supports that the utility of oral fluid samples in

surveillance programs to detect new infections of JE using sentinel pigs and rope-based methods could be useful since the duration of viremia is relatively short, lasting less than five days on average. Further support of oral fluid collection includes the detection of viral RNA in oral fluid up to 14 DPI in domestic pigs (Lyons et al., 2018). This proposes that the shedding of virus through the oral route may aid in vector free spread of JEV, and thus monitoring JEV in oral fluid could be an effective tool for early detection of spread within a swine population (Lyons et al., 2018; Park et al., 2018; Ricklin et al., 2016a). Investigations of vaccines and mAbs against JE for use in swine could be of interest to limit the vector free spread of JE in oral fluid, providing protection to the swine and humans involved in the pork industry.

Nasal swabs obtained over the course of the experiment indicated that the viral load of the isotype control pigs steadily increased, remaining detectable even in the absence of viremia indicating that similar to oral fluid, nasal secretions could contribute to vector free transmission of JEV. Pigs that were passively immunized with either mAb JEV-31 or JEV-169 experienced lower amounts of nasal shedding as well as a reduction in the viral load of the nasal epithelium. While viremia was cleared from the serum in the isotype control pigs at three DPI, the nasal epithelium of these pigs reached its peak titer at three DPI. This supports the use of nasal swabs as a non-invasive, individual animal sample of high diagnostic value, as it would allow for an extended time frame in investigations of clinically ill animals.

Passive immunization of mAb JEV-31 or JEV-169 resulted in lower viral loads detected via fecal swabs compared to the isotype control animals suggesting there was less viral shedding through the digestive tract of the treated pigs. The reduction of fecal shedding indicates that JE vaccines could contribute to herd immunity and help prevent the vector free transmission of JEV through feces, if utilized in swine herds. The use of mAbs to evaluate JE vaccines for swine could

be investigated in the future and potentially be employed to help limit human exposure to infected swine oral, nasal, and fecal secretions. This would reduce the infection risk to individuals who work, own, and handle animals in the event of an outbreak.

Future directions

These experiments looked to improve flavivirus prevention and control measures. Specifically, this included the determination of competent vectors in North America for emerging USUV and assessing JE vaccine protection in the face of shifting JEV genotypes. Two *Culex* species found in North America were identified as being susceptible to infection with USUV and capable of transmission. This suggests the importance of surveillance and control strategies to monitor flaviviruses and prevent their introduction and could include adding USUV to the list of viruses tested in flavivirus panels. This can also involve surveillance of domestic animal hosts, including oral fluid surveillance in swine to detect incidences of JE. Future work could be directed at specific prevention and control methods for mosquitoes susceptible to flaviviruses including larvicides and insecticides. Evaluation of the transmission dynamics of USUV in North American bird species would also aid in improving flavivirus control. Several North American bird species have been identified as competent hosts for WNV as well as JEV, in the event of JEV geographical expansion to North America (Komar et al., 2003; Nemeth et al., 2012). This suggests the importance of evaluating similar species for their competence to USUV infection. Specifically, these evaluations could include North American crows, finches, and sparrows.

Improvements to flavivirus prevention measures were also assessed through the protective effects of two anti-JEV mAbs. This included the potential feasibility of miniature swine for use in evaluating vaccines for potential future use in both animals and humans. The work presented

within this dissertation demonstrated that mAbs derived from a JEV GIII vaccine strain could provide protection against infection *in vivo* from a GI strain. While this work provides insight into the prophylactic use of these anti-JEV mAbs with implications regarding current vaccine efficacy and the potential value of miniature swine for these types of investigations, there were also limitations. It remains to be determined whether these mAbs and the current JE vaccines can provide broad protection *in vivo*, ideally in a relevant large animal model, among multiple genotypes of JEV. Of particular importance would be determining whether the mAbs would be able to protect against other endemic JEV genotypes co-circulating with emerging genotype I strains. While this work demonstrated the feasibility of miniature swine to study JEV, further investigation would be needed to continue to establish their reliability and responsiveness to experimental challenge.

The duration of protection provided by both mAbs needs further elucidation. Variations between strains of JEV could change the effectiveness of a vaccine or therapeutic. For example, such investigations could include evaluating the protection and response of JEV-31 and JEV-169 against the JEV Muar strain (GV) *in vivo* and *in vitro*, not previously assessed. Future work could also look into antiviral treatments for JE by studying mAbs such as JEV-31 and JEV-169 in domestic swine. As current JE treatments are largely supportive, the domestic swine platform described in this dissertation could be utilized to develop remedies against severe JE manifestations and decrease severe neurologic complications.

Another potential application for this work is evaluating monotherapies and antibody cocktails. The use of mAbs for prevention or treatment of viral infectious diseases has increased with advances in technology. For use as a monotherapy or antibody cocktail, antibodies should ideally be potently neutralizing, broadly reactive, and confer specific protection. Antibody

cocktails can have benefits over monotherapies, providing greater efficacy and resistance to viral escape mutants. In this study, vaccination derived anti-JEV mAbs JEV-31 and JEV-169 were evaluated independently, and consequently found to provide protection prophylactically against challenge with JE-91, a GI strain. As these mAbs bind to different epitopes, combining these two mAbs could lessen observed clinical signs even further and prevent resistant variants of JEV.

Therapies are being evaluated for several infectious diseases including influenza virus, Ebola virus, and Zika virus (Gilchuk et al., 2020; Laursen and Wilson, 2013; Stettler et al., 2016). The aforementioned therapies include looking at mAbs, which together, are able to improve virus binding and neutralization activity, aiding in the design of therapeutic cocktails. Similar studies could be done for anti-JEV mAbs to determine cooperative interactions that could be beneficial in continued efforts for JE recovery.

Although there are licensed vaccines for several flaviviruses, outbreaks are still occurring. As treatment options for flaviviruses are largely supportive, the development of improved prevention and control measures is becoming increasingly important. The work provided in this dissertation provides insight into competent vectors for USUV and the protective efficacy of two anti-JEV mAbs against JEV. Further, it highlights the utility of miniature swine for determining JE vaccine efficacy. It will continue to be crucial to evaluate and improve prevention and control measures against flaviviruses, through both vector control and vaccination.

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Appendix A - Statistical Analyses

Table 6-1. Analysis of Table 3-2. Infection, dissemination, and positive detection in saliva of Usutu virus.

Type of data	Categorical
Method	Fisher's exact test
P-value	> 0.05 except for infection of *Cx. pipiens* from 7 DPI to 14 DPI ($p < 0.05$)

Table 6-2. Analysis of Figure 4-1 Individual $PRNT_{50}$ titers at 0 days post infection prior to challenge.

Type of data	Continuous
Data distribution	Non normal
Method	Kruskal Wallis ANOVA
P-value	< 0.05
Post hoc	Dunnett's test

Table 6-3. Analysis of Figure 4-2 Individual temperatures.

Type of data	Continuous
Data distribution	Non normal
Method	Kruskal Wallis ANOVA
P-value	< 0.05 2 DPI and 3 DPI
Post hoc	Dunnett's test

Table 6-4. Analysis of Figure 6-1 Viral titers of serum following JEV challenge in individual pigs.

Type of data	Continuous
Data distribution	Non normal
Method	Kruskal Wallis ANOVA
P-value	< 0.05 2 and 3 DPI
Post hoc	Dunnett's test

Table 6-5. Analysis of Figure 4-4 Viral load in oral fluid.

Type of data	Continuous
Data distribution	Non normal
Method	Kruskal Wallis ANOVA
P-value	> 0.05

Table 6-6. Analysis of Figure 4-5 Viral load in nasal swabs.

Type of data	Continuous
Data distribution	Non normal
Method	Kruskal Wallis ANOVA
P-value	> 0.05 except 3 DPI ($p < 0.05$)
Post hoc	Dunnett's test

Table 6-7. Analysis of Figure 4-6 Viral load in nasal epithelium.

Type of data	Continuous
Data distribution	Non normal
Method	Kruskal Wallis ANOVA
P-value	< 0.05
Post hoc	Dunnett's test

Table 6-8. Analysis of Figure 4-7 Viral load in fecal swabs.

Type of data	Continuous
Data distribution	Non normal
Method	Kruskal-Wallis ANOVA
P-value	> 0.05

Table 6-9. Analysis of Figure 4-8 Viral load in brain tissue.

Type of data	Continuous	
Data distribution	Non normal	Normal (cerebellum)
Method	Kruskal-Wallis ANOVA	One-way ANOVA
P-value	< 0.05	
Post hoc	Dunnett's test	

Table 6-10. Analysis of Figure 4-9 Viral load in lymphoid tissue.

Type of data	Continuous
Data distribution	Non normal
Method	Kruskal-Wallis ANOVA
P-value	< 0.05 for all lymphoid tissue except the mesenteric lymph node ($p > 0.05$)
Post hoc	Dunnett's test

Table 6-11. Analysis of Figure 5-1 Individual $PRNT_{50}$ titers at 0 days post infection prior to challenge.

Type of data	Continuous
Data distribution	Non normal
Method	Kruskal Wallis ANOVA
P-value	< 0.05
Post hoc	Dunnett's test

Table 6-12. Analysis of Figure 5-2 Individual temperatures.

Type of data	Continuous
Data distribution	Non normal
Method	Kruskal Wallis ANOVA
P-value	< 0.05 2 DPI and 3 DPI
Post hoc	Dunnett's test

Table 6-13. Analysis of Figure 5-2 Viral titers of serum following JEV challenge in individual pigs.

Type of data	Continuous
Data distribution	Non normal
Method	Kruskal Wallis ANOVA
P-value	< 0.05 2 and 3 DPI
Post hoc	Dunnett's test

Table 6-14. Analysis of Figure 5-4 Viral load in oral fluid.

Type of data	Continuous
Data distribution	Non normal
Method	Kruskal Wallis ANOVA
P-value	> 0.05

Table 6-15. Analysis of Figure 5-5 Viral load in nasal swabs.

Type of data	Continuous
Data distribution	Non normal
Method	Kruskal Wallis ANOVA
P-value	> 0.05 except 3 DPI ($p < 0.05$)
Post hoc	Dunnett's test

Table 6-16. Analysis of Figure 5-6 Viral load in nasal epithelium.

Type of data	Continuous
Data distribution	Non normal
Method	Kruskal Wallis ANOVA
P-value	< 0.05
Post hoc	Dunnett's test

Table 6-17. Analysis of Figure 5-7 Viral load in fecal swabs.

Type of data	Continuous
Data distribution	Non normal
Method	Kruskal-Wallis ANOVA
P-value	> 0.05

Table 6-18. Analysis of Figure 5-8 Viral load in brain tissue.

Type of data	Continuous	
Data distribution	Non normal	Normal (cerebellum)
Method	Kruskal-Wallis ANOVA	One-way ANOVA
P-value	< 0.05	
Post hoc	Dunnett's test	

Table 6-19. Analysis of Figure 5-9 Viral load in lymphoid tissue.

Type of data	Continuous
Data distribution	Non normal
Method	Kruskal-Wallis ANOVA
P-value	< 0.05 for all lymphoid tissue except the mesenteric lymph node ($p > 0.05$)
Post hoc	Dunnett's test