Susceptibility and disease pathogenesis of North American domestic and feral pigs to Japanese encephalitis virus

by

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B.S., University of Nevada Las Vegas, 2013
D.V.M., Kansas State University, 2020

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

Japanese encephalitis virus (JEV), a mosquito-borne flavivirus capable of causing fatal encephalitis, is maintained in nature between infected mosquitoes and viremic swine and avian species, with humans as a dead-end host. At present, JEV is only endemic to parts of the Asia-Pacific region, but the presence of large numbers of susceptible vertebrate hosts and competent vectors outside its endemic areas is a significant concern in its potential for dispersal into new territories. Previously, North American avian species and *Culex* species mosquitoes have been shown to be susceptible and competent for JEV transmission. A critical but missing gap of knowledge is whether or not the swine species in the United States are also susceptible to JEV. The objective of this dissertation was to address this important research gap and determine the susceptibility profile and pathogenesis of JEV in North American pigs.

Three specific aims were pursued to test the central hypothesis that North American domestic and feral pigs are susceptible to JEV and can potentially support its transmission. In Aim 1, the susceptibility of North American domestic pigs to JEV was determined through the invasive challenge of intravenous inoculation. All pigs became viremic, seroconverted, and developed similar pathologic outcomes as observed in published studies. In Aim 2, our approach was to mimic the natural route of transmission more closely via intradermal inoculation. In the same experiment, mosquito salivary gland extract (SGE) was inoculated with infectious viruses to investigate the effects of mosquito saliva in the disease pathogenesis of JEV. Piglets were simultaneously co-inoculated with JEV and SGE to recapitulate the actual infection route in nature. In contrast to the enhanced virus infection and disease severity reported in mice, the presence of mosquito saliva in the JEV inoculation altered the fever and viral nasal shedding kinetics but, interestingly, did not impact the dynamics and severity of viremia, clinical signs, and

neuroinvasion. Lastly, Aim 3 was conducted to establish a feral pig model for JEV, using the Sinclair miniature research swine that has been bred to have a feral genotypic and phenotypic background. Intradermal JEV challenge of these pigs resulted in high viremia, viral nasal shedding, and systemic dissemination comparable to JEV infection in domestic pigs.

Together, our results indicate that many potential enzootic hosts needed for JEV transmission cycle are present in North America. These findings provided a better understanding of how JEV behaves in its enzootic hosts – the domestic and feral pigs. The work presented in this dissertation provides valuable data and novel animal models of JEV to further our understanding of this significant pathogen and contribute to the development of effective countermeasures to ultimately protect the public health and the agricultural industry.

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Major Professor
Dr. Dana L. Vanlandingham

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Dedication

I would like to dedicate this dissertation to my family. We have our differences but I still love you all. Thanks for your support.

Chapter 1 - Overview of Japanese encephalitis virus

Introduction

The earliest case of Japanese encephalitis (JE) or "summer encephalitis" was described in 1871 in Japan (Hiroyama, 1962). The significance of the disease and its epidemic potential were not highlighted until years later when waves of outbreaks occurred in several Asian countries in the early 20th century (Hiroyama, 1962; Taniguchi et al., 1936; Vasilakis et al., 2019). The first documented large outbreak of JE occurred in 1924 in Japan, which resulted in more than 6,000 cases with a 60% fatality rate (Hiroyama, 1962). In the same year, the filterable agent was isolated from the brain tissue of a human patient using rabbits (Erlanger et al., 2009; Tsai, 1990). However, it was not until 1934 when the isolated virus was proven to be the causative agent of JE by demonstrating the disease through the intracranial challenge of monkeys (Tsai, 1990). Amidst the ongoing outbreaks in Japan in the 1930s, the role of mosquitoes, particularly *Culex tritaeniorhynchus*, in JE transmission was determined and later helped establish the role of water-wading birds and pigs as important amplifying hosts of the enzootic cycle (Erlanger et al., 2009; Hammon et al., 1949; Scherer et al., 1959a; Tsai, 1990). Frequent large outbreaks continued into the 1960s in Japan, Korea, China, and later included parts of Southeast Asia (Hiroyama, 1962; Hullinghorst et al., 1951; Kono and Kim, 1969; Tsai, 1990), earning the description of JE as the "plague of the orient" (Monath, 1988).

While JE cases have significantly decreased from the 1970s through the implementation of vaccine programs, Japanese encephalitis virus (JEV) is still a significant pathogen today that warrants further ongoing research for the development of efficacious preventative and therapeutic

treatments. Japanese encephalitis virus is currently the leading viral cause of fatal pediatric encephalitis in the Asia-Pacific region (Centers for Disease Control and Prevention, 2019; World Health Organization, 2019). Although efficacious vaccines are available, approximately 68,000 cases are still reported each year in the endemic countries, of which about 75% occur in children under 15 years of age (Campbell et al., 2011; World Health Organization, 2019). An estimate of 3 billion people are at risk of JEV infection in 24 endemic countries (Erlanger et al., 2009; World Health Organization, 2019). While primarily recognized as a human pathogen, JEV also has veterinary and agricultural importance because it is capable of causing reproductive diseases in pigs. Currently, JEV is a notifiable disease to the World Organization for Animal Health (OIE) and is listed as a high-priority biosafety level 3 pathogen for biodefense and emerging disease research by several federal agencies (National Institute of Allergy and Infectious Diseases, 2018; Yang, 2019).

The potential dispersal of JEV into new geographic areas is, therefore, an important public and veterinary health concern. While JEV is currently endemic to large parts of Asia and the Pacific region, the presence of competent arthropod vectors and susceptible amplifying vertebrate hosts outside its current geographic distribution could potentially lead to the dispersal and subsequent establishment into new territories. Although the disease has not been reported in North America, a proactive approach in identifying the susceptible arthropod and vertebrate species and understanding the consequences of their infection is critical to assess risk and develop effective countermeasures against a potential introduction and the establishment of a JEV transmission cycle in a new region. It would be nearly impossible to present the entire biology of JEV, so for our purpose the literature review focused on topics relevant to understanding and exploring this subject of dispersal and emergence potential of JEV in North America. Below is a general overview of the

etiological agent of JE with attention to the following topics: clinical disease, transmission cycle, diagnostic techniques, vaccines, geographic distribution, and pathogenesis.

Classification of the virus

The International Committee on Taxonomy of Viruses classifies JEV as an enveloped RNA virus in the *Flaviviridae* family under genus *Flavivirus* (International Committee on Taxonomy of Viruses, 2021). The *Flaviviridae* family is one of the major families of arthropod-borne viruses (arboviruses) and contains several important human or zoonotic pathogens including dengue viruses and yellow fever virus (Simmonds et al., 2017). By definition, arboviruses are "viruses which are maintained in nature principally, or to an important extent, through biological transmission between susceptible vertebrate hosts by hematophagous arthropods; they multiply and produce viremia in the vertebrates, multiply in the tissues of arthropods, and are passed on to new vertebrates by the bites of arthropod after a period of extrinsic incubation" (World Health Organization, 1967). At present, there are four genera within the *Flaviviridae* family: *Hepacivirus, Pegivirus, Pestivirius*, and *Flavivirus* (Kemenesi and Bányai, 2019). More than 70 members belong in the *Flavivirus* genus, which is composed of viruses that can replicate in a diverse range of invertebrates and vertebrate cells (Clark et al., 2012; Cook and Holmes, 2006). Flaviviruses can be mosquito-borne, tick-borne, or have no known arthropod vectors and with or without mammals and birds as the primary hosts (Kemenesi and Bányai, 2019). There are also insect-only flaviviruses (International Committee on Taxonomy of Viruses, 2021). More than 50% of the known flaviviruses can be pathogenic to humans and/or animals, capable of causing a range of clinical signs from asymptomatic to severe diseases including hemorrhagic fever, shock, or neurologic disease (International Committee on Taxonomy of Viruses, 2021).

Prior to genetic sequencing becoming available, flaviviruses were arranged into antigenically distinct serological complexes or serocomplexes based on serum cross neutralization assays. Japanese encephalitis virus belongs to the JE serocomplex, which contains the largest number of mosquito-borne viruses associated with neuroinvasive disease including West Nile virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, and Usutu virus (Murphy et al., 1999; Schweitzer et al., 2009). The majority of its members have enzootic cycles primarily involving birds and cause diseases in epizootic spillovers to humans and other susceptible vertebrate hosts (Schweitzer et al., 2009).

Clinical disease

Clinical outcomes in human patients

Japanese encephalitis virus is the most important cause of viral encephalitis in Asia, recognized for its potential to cause fatal infections of the central nervous system (World Health Organization, 2019). Japanese encephalitis disease is mainly observed in immunologically-naïve individuals in endemic areas. Clinical symptoms in infected individuals can range from nonspecific flu-like symptoms including headache, high fever, and lethargy to severe clinical manifestations such as paralysis, motor and memory deficits, and seizures (Ghosh and Basu, 2009; Monath, 2002). Young children are more commonly affected (Campbell et al., 2011). In infants or young children, initial symptoms may also include anorexia, nausea, abdominal discomfort or pain, vomiting, diarrhea, and/or acute convulsions or seizures (Tsai, 1990). The incubation period usually ranges between 5 to 15 days with illness first starting with a high fever, change in mental status, and gradual decline in speech or motor functions (Tsai, 1990). Generalized weakness, hypertonia, and hyperreflexia are usually the most common motor abnormalities reported in early

JE patients (Tsai, 1990). Although these symptomatic infections are rare, mortality rates from Japanese encephalitis cases can reach 30%, leading to an estimate of 10,000 to 15,000 deaths every year (Campbell et al., 2011; Chen et al., 2015). Additionally, up to 50% of the survivors may suffer from serious permanent neurologic disabilities such as cognitive, motor, and coordination abnormalities, further increasing the public health burden in the affected countries (Hegde and Gore, 2017; Tsai, 1990; World Health Organization, 2019). Poor prognosis is usually associated with clinical signs such as short prodromal period (time between initial symptoms to full disease), prolonged fever, deep obtundation, respiratory dysfunction, and status epilepticus (Tsai, 1990). Presence of high virus concentrations and low antibody titers in the cerebrospinal fluid, which are reflective of uninhibited viral proliferation in the brain, are also highly associated with fatal outcome (Tsai, 1990).

Although very rare, JEV infection has also been associated with abnormalities in human pregnancy (Chaturvedi et al., 1980; Mathur et al., 1985; Tsai, 1990). This was documented when JEV was first introduced into northern India (Tsai, 1990; Tsai, 2006). In a series of outbreaks in Uttar Pradesh, India between 1978 and 1980, women who became infected with JEV in the first or second trimesters miscarried and the virus was isolated from the placenta and fetal brain (Tsai, 1990). However, those who were infected with JEV in the third trimester did not experience any adverse pregnancy outcomes (Tsai, 1990). The occurrence of congenital infections of JEV is most likely rare because, where JEV is endemic, children are either exposed or vaccinated and become immune at an early age so very few young women may be at risk of infection at or during pregnancy (Tsai, 1990; Tsai, 2006). This rare reproductive outcome could potentially become more apparent in an immunologically-naïve human population in a new territory, but this is only a speculation.

Clinical outcomes in infected swine

Although primarily known as a human pathogen, JEV is also an agricultural pathogen that can greatly impact the swine and pork industry (Ghosh and Basu, 2009). Clinical disease in pigs caused by JEV infection is age-specific. Infection in sexually matured adult pigs can result in reproductive failure in forms of abortions and transient infertility (Desingu et al., 2016; Joo, 1981; Shimizu et al., 1954; Takashima et al., 1988). Abortions, abnormal farrowing, mummified fetuses, and weak piglets are most commonly observed if the pregnant sow became infected before 60 to 70 days of gestation (Platt and Joo, 2006; Shimizu et al., 1954). Reports estimate that approximately 40% to 53% of unvaccinated pregnant sows had stillbirths and abortions in Japan during the epidemic seasons between 1947 and 1969 (Fujisaki, 1971; Imoto et al., 2010; Shimizu and Kawakami, 1949). Reproductive disease from JEV infection can also affect boars. Infected boars can develop edematous or congested orchitis with abnormal spermatozoa but are capable of recovering completely most of the time (Hashimura et al., 1976; Ogasa et al., 1977).

In young pigs, JEV can invade the central nervous system similar to human cases and cause nonsuppurative meningoencephalitis (Yamada et al., 2004). However, natural infection and disease of young piglets are not commonly reported from the endemic areas, possibly due to the presence of maternal antibodies which can last up to 6 months of age (Platt and Joo, 2006; Scherer et al., 1959b). While non-specific clinical signs such as fever, anorexia, and depression are observed early with JEV infection, neurologic signs such as hind limb tremors or ataxia can sometimes develop after 5 days post-infection (Fujisaki, 1975; Kodama et al., 1968; Yamada et al., 2009). Some infected pigs can progress into developing a wasting-like syndrome (Yamada et al., 2004; Yamada et al., 2009). Nonetheless, JEV infection in pigs is generally not considered a

lethal swine disease. Most pigs survive, seroconvert after infection, and are even capable of developing secondary JEV infections when infected with a different genotype under laboratory conditions (García-Nicolás et al., 2017). However, pigs are important in the JEV transmission cycle because infected pigs can also pose a further threat to human and public health because they are capable of developing high viremia and thereby contribute to the maintenance of the transmission cycle.

Transmission cycle

Understanding how a virus is transmitted and maintained in nature is an important part of infection risk mitigation. The transmission cycles of arthropod-vectored viruses involve a complex interaction amongst the hematophagous vectors, virus, vertebrate host, and environment. This is complicated further by the several separate factors that impact each of these components that significantly influence the disease outcome, as summarized in **Figure 1.1** (Anez et al., 2012; Chouin Carneiro and Dos Santos, 2017). Therefore, how a specific emerging arbovirus and their eventual outbreaks might behave, especially in new territories, can be fairly complicated. Different types of transmission cycles can occur in a location depending on the type of hosts (i.e. wild, domestic, or human) and vectors (i.e. urban, primary, or accessory) involved: enzootic or sylvatic cycle in the wild, epizootic or rural cycle, and epidemic or urban cycle involving human cases (Anez et al., 2012; Go et al., 2014). The enzootic or sylvatic cycle is the natural transmission of the virus between the wild vertebrate hosts and primary or enzootic arthropod vectors that leads to the amplification and maintenance of the virus in nature (Go et al., 2014). The epizootic or rural cycle is the transmission of virus between the domestic animals and primary or accessory arthropod vectors (Go et al., 2014). This usually leads to viral outbreaks in the domestic animal

population in which the virus is amplified and often has implications extending to the humans by the arthropod vectors. Lastly, the epidemic or urban cycle is the virus cycle between humans as the source of infection due to being capable of developing high level of viremia and the urban arthropod vectors (Go et al., 2014). With humans being dead-end or incidental hosts for JEV, meaning that they cannot produce sufficient viremia capable of infecting mosquitoes, JEV only has enzootic and epizootic transmission cycles, as shown in **Figure 1.2**. Out of these, the enzootic transmission cycle is the most important to understand for our purpose because it is responsible for viral maintenance in a specific geographic region.

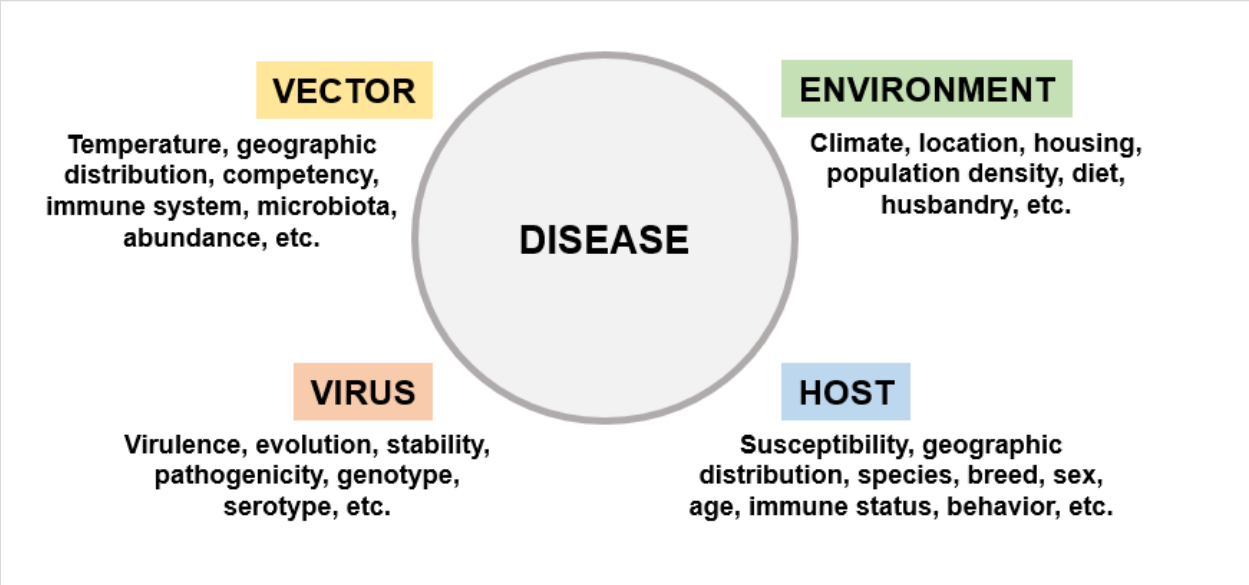

Figure 1.1. Complex relationships in arbovirus transmission and disease outcome.

Japanese encephalitis is primarily a rural agricultural disease (Tsai, 1990). It is maintained in mosquito to bird or pig to mosquito cycle with incidental transmission to humans through bites from infected *Culex* species mosquitoes (Buescher et al., 1959). Pigs and birds are the natural amplifying hosts of JEV, which means that they develop high and often prolonged duration of

viremia capable of infecting arthropod vectors. Most epidemics in Asia are driven by the close association between amplifying hosts and humans with mosquitoes linking the two types of hosts: (1) domestic pigs and humans in backyard pig farming and (2) water-wading birds and humans in rice paddy fields (Le Flohic et al., 2013; Tsai, 1990). The susceptible avian and swine species also play important roles in enzootic transmission in the wild, establishing the endemic status to a location. There are many other vertebrate species that are susceptible to JEV including cattle, goats, horses, dogs, bats, reptiles, and chickens (Cleton et al., 2014; Ghosh and Basu, 2009; Gould et al., 1964; Murphy et al., 1999). However, most are dead-end hosts and only those that are capable of developing high level of viremia sufficient to infect competent mosquitoes are important in the natural transmission cycle of the virus.

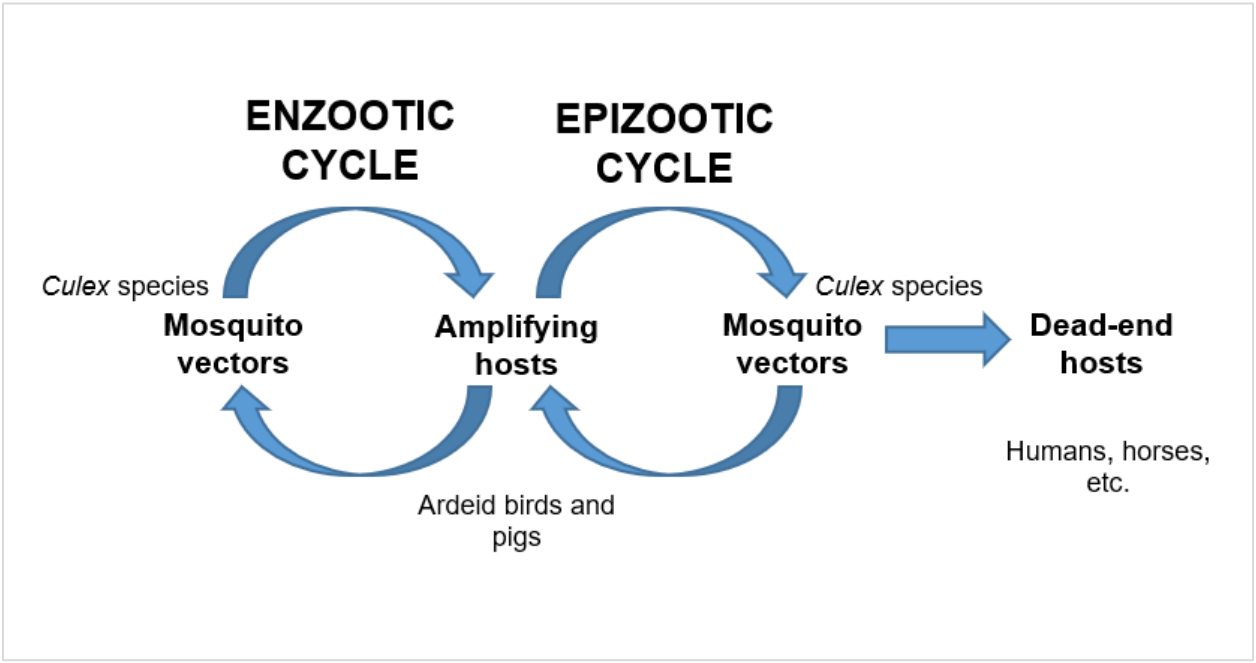

Figure 1.2. Transmission cycles of Japanese encephalitis virus.

Amplification hosts

Avian species

Birds play one of the central roles in the transmission cycle of JEV due to their development of high viremia and migratory behaviors that are important for viral maintenance and dispersal. Over 90 bird species are known to be potential amplifying and reservoir hosts of JEV (Le Flohic et al., 2013). Wild water-wading birds, especially egrets and herons of the *Ardeidae* family, are highly susceptible to JEV infection (Boyle et al., 1983; Le Flohic et al., 2013). Many of them are widely distributed, migratory, and thereby are suspected to be potentially responsible for the dispersal of JEV to new geographic regions (Le Flohic et al., 2013; Solomon et al., 2003; Yang et al., 2011). Migratory birds, in general, play a significant role in the ecology and circulation of emerging infectious diseases, often leading to the dispersal and establishment of new endemic locations along the migration routes (Georgopoulou and Tsiouris, 2008; Reed et al., 2003). Each fall, billions of birds travel through the different major global flyways that connect the north and south hemispheres (BirdLife International, 2021; Reed et al., 2003). Migratory birds have been identified as potential long-distance vehicles for many zoonotic diseases including viruses such as West Nile virus (Owen et al., 2006; Reed et al., 2003), Usutu virus (Engel et al., 2016), and influenza A virus (Reed et al., 2003; Webby and Webster, 2001; Zhang et al., 2014) as well as enteropathogens (Hudson et al., 2000; Reed et al., 2003). Therefore, migration of susceptible birds can likely be a potential driver in the dispersal of JEV.

The black-crowned night heron (*Nycticorax nycticorax*) is implicated to be involved in the primary cycle of JEV and thereby plays a significant role in the maintenance and transmission of JEV (Buescher et al., 1959; Solomon et al., 2003). In JEV endemic regions, neutralizing maternal antibodies in these birds usually wane by 3 weeks of age and they become fully susceptible

afterwards (Scherer et al., 1959a). Infected birds are asymptomatic. Upon infection, maximum viral titers are generally higher in young birds (i.e. 2- to 5-months of age producing approximately 10^4 to 10^5 mouse median lethal dose [LD_{50}]/ml) compared to older birds (i.e. greater than 8-months of age producing 10^3 to 10^4 mouse LD_{50}/ml) (Boyle et al., 1983). Viremia in these birds generally last 3 to 5 days after becoming detectable at 1 or 2 days post-infection (Boyle et al., 1983). The Asiatic cattle egret (*Bulbulcus ibis coromandus*) is another important migrating bird that may contribute to the maintenance of JEV transmission (Solomon et al., 2003).

Swine species

Domestic pigs (*Sus scrofa*) are another amplifying host of JEV and may play a more significant role than birds in the ecology of JEV due to the close interactions with humans through farming and agriculture. Numerous JEV isolates have been detected in infected pigs (Desingu et al., 2016; Ladreyt et al., 2019; Yamada et al., 2004). In contrast to birds, infected pigs can develop pathologic outcomes, as described in a previous section. Under laboratory conditions, pigs infected with JEV can reach peak viremia titers of approximately 10^4 median tissue culture infectious dose ($TCID_{50}$)/ml at 2 or 3 days post-infection with evidence of viral tissue dissemination and neuroinvasion by that time point (Ricklin et al., 2016a; Ricklin et al., 2016b; Young et al., 2020). Viremia is transient and generally becomes undetectable after 4 to 5 days post-infection (Ricklin et al., 2016a). Interestingly, previous infection with one genotype virus may not fully prevent a secondary infection with another genotype in experimental settings (García-Nicolás et al., 2017). Although not as robust, the heterologous infection can still lead to detection of low viral RNA loads in lymphoid or nervous tissues and in oronasal swabs within 10 days post-infection (García-Nicolás et al., 2017). Additionally, pigs with neutralizing antibodies against JEV were documented

to still be susceptible to JEV infection by mosquito bites and developed viremia of sufficient magnitude to infect a low percentage of biting mosquitoes (Hurlbut, 1964).

The majority of all modern pig breeds have derived from the wild Eurasian boar (still classified as *Sus scrofa*), with domestication dating back approximately 7,000 to 9,000 years ago (Gutierrez et al., 2015). Much like their domestic counterparts, wild boars have been identified as relevant drivers of JEV outbreaks in the endemic areas (Ruiz-Fons, 2017). By definition, wild or feral boars are a group of *Sus scrofa* biotypes that includes feral or escaped domestic pigs, Eurasian or Russian wild boars, and their cross-bred hybrids (Ruiz-Fons, 2017). High seroprevalence of JEV, ranging from around 66% to 83% in some regions (Nidaira et al., 2014; Nidaira et al., 2007; Ohno et al., 2009; Yang et al., 2012a), and RNA isolation (Nidaira et al., 2008) have been documented in wild boars sampled from Korea and Japan. However, despite their presumed importance as a JEV enzootic host, how JEV impacts feral pigs in terms of clinical signs or pathology remains undetermined.

Arthropod vectors

The mosquito (Order Diptera, Family *Culicidae*) plays the critical role of arthropod vector in the mode of JEV transmission. Japanese encephalitis virus has been isolated from a variety of field-collected specimens of different *Culex*, *Aedes*, *Anopheles,* and *Mansonia* species as well as in midges (*Lasiohelea taiwana*) and even in ticks (*Haemaphysalis japonica*) (Le Flohic et al., 2013; Rosen, 1986). However, not all of them have epidemiological and/or ecological importance. For example, the isolation of JEV in these arthropods does not exclude the possibility of detection due to recent engorgement of viremic blood. The insect may also not be susceptible to the arboviral infection. This susceptibility is necessary for the virus to replicate in the arthropod and disseminate

into the salivary glands for eventual transmission of the virus. For the arthropod vector to be of importance, it must be a competent vector. By definition, competent vector species must be capable of acquiring the virus infection in nature, transmit the infection by feeding on susceptible vertebrate hosts, and be abundant enough to be significant (Rosen, 1986). The major vectors that fit these criteria include *Culex tritaeniorhynchus*, *Cx. gelidus*, *Cx. vishnui*, and *Cx. annulirostris* (Le Flohic et al., 2013; Rosen, 1986). From this short list, *Cx. tritaeniorhynchus* is recognized as the principal vector of JEV due to its high susceptibility, transmission rate, and wide distribution (Clark et al., 2012; Gresser et al., 1958; Rosen, 1986). *Culex* species mosquitoes generally use ground pools, especially flooded rice paddies, for surface egg-laying and larval habitat (Tsai, 1990). They are most active and feed during the crepuscular periods (Tsai, 1990). They are usually primarily exophilic, seeking hosts outdoors, and zoophilic, preferring animals to humans for feeding (Tsai, 1990). Of these species, *Cx tritaeniorhynchus*, in particular, have a predilection to feed on pigs, further supporting the role of pigs as important amplifying hosts (Misra and Kalita, 2010). These behaviors also highlight the importance of *Cx. tritaeniorhynchus* as primary vectors in the enzootic transmission cycle of JEV.

Virus replication in the arthropod vector is crucial for the maintenance and transmission of the arbovirus. The 50% infective dose for *Cx tritaeniorhynchus* is between 2 to 3 log mouse LD_{50} (Hurlbut, 1964) but viremia as low as 1 log mouse LD_{50} has been documented to be capable of infecting a low percentage of feeding mosquitoes (Gould et al., 1964; Sasaki et al., 1982). Following a viremic blood meal, the virus titer in the mosquito midgut lumen must be high enough to overcome the midgut barrier and immune system, infect the gut epithelial cells, and disseminate into the haemocoel (Hardy et al., 1983). The virus then disseminates and infects the salivary glands so that it is excreted with salivary components when the mosquito feeds upon the next host (Hardy

et al., 1983). An average of 5 log focus-forming units (FFU)/ml of JEV can be detected in the saliva of infected *Cx. tritaeniorhynchus* (Faizah et al., 2021), but the transmitted dose can range from 1 to 7 log plaque forming units (PFU) of virus (Schneider and Higgs, 2008). However, successful blood feeding is not necessary as mosquito can also transmit virus simply by probing (Gresser et al., 1958; Styer et al., 2006). Non-viremic transmission, or non-replicative transmission, is also possible in which mosquitoes get infected by feeding nearby an infected mosquito on a vertebrate host, whether amplifying or incidental and/or already immune to the virus of interest (Higgs et al., 2005; Huang et al., 2019). Additionally, vertical transmission of JEV in mosquitoes, either transovarial or at oviposition, has been demonstrated under laboratory conditions for several known vectors including *Cx. pseudovishui*, *Cx. tritaeniorhynchus*, *Cx. annulus*, *Cx. quinquefasciatus*, *Armigeres subalbatus*, *Aedes albopictus*, and *Aedes togoi* (Mourya et al., 1991; Rosen et al., 1989; Rosen et al., 1978). The real ecological role is unknown. In summary, the presence of both competent vectors and amplifying hosts are necessary for the enzootic transmission of JEV. The presence of JEV competent mosquitoes in a region is what mainly drives disease transmission, but susceptible amplification hosts are necessary to sustain the viral cycle.

Other modes of transmission

Although JEV is primarily transmitted by bites from infected mosquitoes, mucosal transmission could be theoretically possible in humans based on the recent evidence of oral shedding of viral RNA in JE patients detected via throat swab sampling (Bharucha et al., 2018). Laboratory workers, in particular, are in high occupational risk for mucosal or aerosol exposure of high virus concentrations (AJTMH, 1980; Hills et al., 2019). There have been at least 22

laboratory-acquired JEV infections reported thus far (AJTMH, 1980; Hills et al., 2019). Additionally, there has been documented experimental infections of several mammalian hosts, such as macaques, mice, guinea pigs, and pigs, with JEV through the oronasal or intranasal route (Fujisaki, 1975; García-Nicolás et al., 2018; García-Nicolás et al., 2017; Li et al., 2012; Myint et al., 2014; Ricklin et al., 2016a; Yamada et al., 2009). For example, Ricklin et al. (2016a) demonstrated that pigs are highly susceptible to oronasal infection with viral titers as low as 10 $TCID_{50}$ and are also susceptible to nose-to-nose contact with infected pigs via viral nasal secretions, resulting in systemic infection, oronasal viral shedding, and antibody production comparable to needle challenge. In another study, even the empirically-derived live attenuated JEV SA14-14-2 vaccine caused systemic infection, oronasal viral shedding, histopathologic lesions in the brain and lungs, and antibody response after intranasal inoculation in mice (Chai et al., 2019). Additionally, in a recent study by Chai et al. (2019), direct intranasal, contact, and aerosol transmissions between infected and naïve mice were also experimentally demonstrated, prompting the authors to hypothesize that such potential route of transmission may be possible in humans in densely populated endemic regions. Although not as efficacious as the traditional needle routes, mice can be immunized orally with live JEV to induce a productive immune response (Ramakrishna et al., 1999). A conjunctival route of JEV transmission has recently been documented in mice (Sethi et al., 2019).

These studies further highlight the potential of non-vector transmission in nature and are consistent with the observation that mucosal exposure, whether accidental or experimental, can lead to systemic infection. Intranasal administration, for example, is considered a delayed form of intracranial inoculation for encephalitic viruses and can lead to viral dissemination to the brain via the olfactory neurons (Monath et al., 2000). However, a lot of these studies were conducted in

research settings. No true clinical cases of vector-free transmission have been reported to date. The accumulating body of evidence that JEV can be detected in body fluid samples other than blood may also be consequences of the availability of more sensitive methods to detect the presence of nucleic acids. Therefore, the epidemiologic importance of mucosal routes of transmission needs further investigation. Nonetheless, it is important to be aware of these additional, although relatively uncommon, routes of infection to aid in clinical or field diagnosis of JEV disease.

Diagnostic methods

Diagnosis in human patients

Japanese encephalitis diagnosis in infected humans based on patient history and clinical symptoms is generally unreliable due to the initial non-specific symptoms and the list of other more common differential diagnoses based on locality. In North America, the common differential diagnoses for infectious encephalitis in children include herpes simplex virus, enterovirus, human parechoviruses, and arboviruses including La Crosse, West Nile, Eastern equine encephalitis, Powassan, and St. Louis encephalitis viruses (Messacar et al., 2018). The diagnosis can be further complicated by cross-reactivity issues in serology as some of these agents, particularly the flaviviruses, are closely related to each other. Therefore, laboratory diagnosis and confirmation are ultimately necessary. The test recommended by the World Health Organization (WHO) for JEV diagnosis is anti-JEV antibody capture enzyme-linked immunosorbent assay (JEV MAC-ELISA) to detect anti-JEV IgM in cerebrospinal fluid or serum samples (Bharucha et al., 2018; World Health Organization, 2019). This test is capable of detecting up to 75% of JE-positive patients within the first four days after onset of symptoms and almost 100% of JE-positive patients after a week of illness (Tsai, 1990). However, it requires trained professionals, appropriate resources, and

several hours for results (Bharucha et al., 2018). Four-fold or greater change in antibody titer based on hemagglutination inhibition (HI), enzyme-linked immunosorbent assay (ELISA), and/or neutralization assays can also be used as confirmatory tests (Tsai, 1990). One of the biggest challenges for these serologic tests is to have paired samples. False positive results can also occur from cross-reactive heterologous flavivirus infection, but this can be differentiated with further testing such as with epitope blocking ELISA (Kitai et al., 2007; Tsai, 1990). Virus isolation on cell culture and viral genome detection methods, such as TaqMan one-step quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR) assay targeting nonstructural protein 5 (Pyke et al., 2004; Williams et al., 2001) or the 3′ non-translated region (Yang et al., 2004), could further help circumvent the problem of cross-reactive antibodies. However, RNA detection has a short window of time in which it can be a useful tool for human diagnosis.

Currently, most endemic countries conduct some form of national JE surveillance and/or perform subnational surveillance in risk areas with sentinel animal monitoring (Heffelfinger et al., 2017). While JE is reportable to WHO, reporting is highly variable and incomplete due to the varying intensity and quality of JE surveillance and the availability of diagnostic laboratory testing throughout the world (Campbell et al., 2011). For example, in countries where few or no cases have ever been reported prior to 2000, such as Indonesia, Philippines, and Malaysia, hospital-based studies revealed JE to be the true etiology in 17% to 50% of hospitalized encephalitis cases, which were not reflected in the official notifications (Tsai, 2000). As such, the true global incidence of JE may be significantly underestimated and warrants a better reporting system (Campbell et al., 2011; Tsai, 2000).

Diagnosis in infected swine

Infection of swine or birds generally follows closely in time with the onset of detectable mosquito infection but precedes human infection by several weeks (Buescher et al., 1959; Scherer et al., 1959a). Domestic pigs are, thus, considered a good sentinel animal for JEV surveillance. Diagnosis of JEV in pigs can be based on virus isolation in the brain post-mortem (i.e. via cell culture or intracranial challenge of suckling mice), detection of specific IgM and IgG antibodies in cerebrospinal fluid or serum samples via ELISA, and viral RNA detection in a variety of samples such as brain, blood, placental tissues, and cerebrospinal fluid (World Organization for Animal Health, 2019; Yang, 2019). Other serology-based tests to further confirm exposure or active infection include virus neutralization assay, HI test, and complement fixation test (World Organization for Animal Health, 2019; Yang, 2019). However, these tests can be hindered by the presence of maternal antibodies, which may remain detectable until 6 months of age (Scherer et al., 1959b). A less invasive and easier alternate is the rope-based method to collect and sample oral fluids from pig pens to detect JEV RNA from the oronasal secretions within 7 days of infection (Lyons et al., 2018). However, this method has not been tested in the field yet.

Accurate diagnostic capability is critical to detect the presence of the virus in a new territory, but equally important is to understand and review the currently available arsenal in place to combat against this disease in the event of potential virus introduction.

Vaccines

Human vaccines

At present, there are no approved antivirals or specialized therapies available to treat JE. With no specific treatments available, the prevention of JE via vaccination is key (World Health

Organization, 2019). WHO highly recommends the integration of JE vaccines into the national immunization schedules in countries where the disease is a public health priority (Heffelfinger et al., 2017; World Health Organization, 2019). There are currently several inactivated and live attenuated licensed vaccines available as intramuscular or subcutaneous doses that can provide effective means of protection from infection and subsequent disease (Chen et al., 2015; Hills et al., 2019). As summarized in **Table 1.1**, the three types of licensed vaccines available at this time are: (1) inactivated Vero cell-derived vaccines, (2) live attenuated vaccine, and (3) live chimeric vaccine (Chen et al., 2015; Hegde and Gore, 2017; Hills et al., 2019).

Table 1.1. Currently available human vaccines for JEV.

Vaccine types	Vaccine names (virus strain)	Dose regimen
Inactivated Vero cell-derived vaccine (JE-VC)	JEIMMUNUGEN, TC-JEV, or JE-BIK-V (Beijing-1)	Three doses (day 0, 7, and 28) at 12-24 months of age; Booster after 12-14 months and every 3-5 years
	IXIARO, JESPECT, or JEEV (SA14-14-2)	Two doses (day 0 and 28) as early as 2 months of age; Booster after 1 year
Live attenuated vaccine	CDJEVAX (SA14-14-2)	Single dose at 8-9 months of age; Booster after 3-12 months and at 6-7 years of age
Live chimeric vaccine	IMOJEV (ChimeriVax-JE)	Single dose with booster after 5 years for those >18 years old; Single dose with booster at 12-24 months for those between ages of 9 months to 18 years old

References: (Chen et al., 2015; Hegde and Gore, 2017)

Although efficacious products, there are some shortcomings and disadvantages depending on the type of vaccine. With inactivated JEV vaccines, one of the major problems is the requirement of multiple doses and boosters to achieve adequate durable protection, making

vaccination programs costly and potentially compromising compliance (Fei-fei et al., 2008; Lin et al., 1998; Tsai, 2000). There are also concerns of potential allergic or hypersensitivity reactions to certain components of its formulation, such as protamine sulfate, thimerosal, gelatin, and proteins of neural origin and/or rodent origin (Chen et al., 2015; Hegde and Gore, 2017; Tsai, 2000). Another concern is the induction of an incomplete neutralizing antibody profile due to the alteration of antigenicity and immunogenicity of some neutralizing epitopes by formalin inactivation (Fan et al., 2015). Although the product is well tolerated, there are still safety concerns in their production because they are produced from infectious virulent strains and require the appropriate containment facilities that subsequently increase the cost of the product (Imoto et al., 2010).

In contrast, live attenuated vaccines can induce strong humoral and cellular immunity by mimicking natural infection and are capable of eliciting long protective immunity from a single dose (Tsai, 2000; Wang et al., 1999; World Health Organization, 2005; Zheng et al., 2020). However, despite the excellent safety record (Hills et al., 2019; World Health Organization, 2005), there is an inherent risk of reversion to virulence as an empirically derived strain (Fan et al., 2010; Hegde and Gore, 2017; Yang et al., 2014a). For example, serial passage of SA14-14-2 in suckling mouse brain resulted in emergence of adaptive mutations and increased virulence in mice (Yang et al., 2014a). Potential reversion was also observed in pigs vaccinated with SA14-14-2 in China. Isolates from the cerebrospinal fluid of aborted fetuses or stillborn piglets that were randomly collected in piggeries in central China were very closely related to the SA14-14-2 vaccine strain based on phylogenetic analysis of the E gene and were demonstrated to be fatal to suckling mice (Fan et al., 2010; Hegde and Gore, 2017).

Nonetheless, vaccination has been proven to be the most cost-effective measure to protect

people against JEV, significantly reduce JE incidence rates, and decrease the public health burden of JE in the endemic countries (Chen et al., 2015; Hills et al., 2019; Tsai, 1990; Tsai, 2000). Before the production and widespread use of JE vaccines, more than several million cases of JE were reported from East Asia with the highest risk areas having incidence rates as high as 20 cases per 100,000 children per year prior to the 1970s (Campbell et al., 2011; Gao et al., 2014; Hegde and Gore, 2017; Hills et al., 2019; Tsai, 2000). Now, in countries with long-standing and high-quality vaccination programs such as Japan, Korea, China, and Taiwan, the annual incidence rates have decreased to less than one case per 100,000 children (Campbell et al., 2011; Gao et al., 2014; Hills et al., 2019). Economic analysis of Thailand and Shanghai, China demonstrated that the implementation of vaccination programs was estimated to save $73,000 U.S. dollars per one prevented case in treatment cost, disability care, and future lifetime earnings in Thailand (Chen et al., 2015; Siraprapasiri et al., 1997) or up to $500,000 U.S. dollars per 100,000 persons from treatment cost of neurologic sequelae from JEV infection in China (Chen et al., 2015; Ding et al., 2003). A more comprehensive cost-effective analysis of JEV immunization programs for 14 endemic countries demonstrated that vaccination would result in a decrease of approximately 190,000 cases, reduction of close to 6,600,000 disability-adjusted life years, and savings of about $19 million U.S. dollars in acute case hospitalization costs in a period of 14 years (Hegde and Gore, 2017; Suraratdecha et al., 2007).

With support from the PATH (Program for Appropriate Technology in Health) and Gavi Vaccine Alliance international organizations, additional countries, especially those of low-income with high burden of JE, have received support for the introduction of JE vaccines and for improved surveillance systems (Gavi, 2020; PATH, 2018). Approximately more than 300 million children have been successfully vaccinated with the support from PATH, Gavi, and their partners (Gavi,

2020; PATH, 2018). It is estimated that between 2000 and 2015, approximately 308,000 JE cases were averted due to vaccination globally (Quan et al., 2020). However, an estimate of 68,000 annual JE cases affecting mainly young children still occur worldwide (Campbell et al., 2011). Therefore, the increase of vaccine coverage is still needed to maintain control of this disease. Further understanding of JEV and its pathogenesis through animal models or closer mimicry of the natural infection process can also help in the development of therapeutics such as antiviral drugs.

Swine vaccines

Despite the potential for reproductive disease and JEV-infected pigs as a source for epizootic spillover, there are currently no licensed JEV vaccines for pigs. There are only regionally approved vaccines, including live attenuated at222, ML17, and anyang300 vaccines, that are available for local use in Japan, China, and Korea (Fan et al., 2013; Fujisaki, 1975; Lee et al., 2012; Nah et al., 2015). While not licensed for swine use, the live attenuated SA14-14-2 human vaccine is also commonly adopted to immunize swine herds in China at the recommended dose of 10^5 PFU with a booster in 3 to 4 weeks (Fei-fei et al., 2008; Wei et al., 2019; Xu et al., 2004). However, even though the SA14-14-2 vaccine has an excellent safety record for human use, reversion to virulence may be a concern when the vaccine is used in amplifying host like pigs because virus isolates closely related to the SA14-14-2 vaccine strain were detected from the cerebrospinal fluid of aborted fetuses or stillborn piglets from some vaccinated pigs in China (Fan et al., 2010). Additionally, while most of these vaccines are live attenuated vaccines that should elicit robust immune response based on their vaccine type, all of them require boosters to elicit adequate protection (Nah et al., 2015). Even the locally commercialized formalin-inactivated

vaccines also provide inadequate immune response with the addition of boosters (Konishi et al., 2000). As a result, vaccinating pigs against JEV is hard to practice because of costs associated with multiple shots. More research is, therefore, warranted to protect an immunologically naïve population of pigs from JEV infection and disease.

A comprehensive census of pigs between 2007 and 2009 in Bangladesh demonstrated that JEV infected about 20% of susceptible pigs annually (Khan et al., 2014). Based on their SEIR (Susceptible-Exposed-Infective-Recovered) model, vaccinating almost 50% of pigs each year (with assumed 95% vaccine efficacy based on SA14-14-2 efficacy in humans) would result in an estimated 82% reduction in annual incidence in pigs (Khan et al., 2014). Even with a vaccine with 50% efficacy, a significant reduction of 53% in infection incidence of pigs is expected (Khan et al., 2014). As such, improvements of the current vaccine platforms and/or development of novel vaccine candidates for swine use could still be an effective countermeasure to protect the animals from reproductive or neurologic disease that could negatively impact the agricultural industry. However, the cost associated with the vaccine and practice may be a big challenge. Recent swine vaccine research and development for JEV include the use of DNA vaccines (Imoto et al., 2010; Konishi et al., 2000), recombinant pseudorabies virus vector (Xu et al., 2004), lentiviral vector (García-Nicolás et al., 2017), and virus-like particles (Fan et al., 2018) to deliver immunogenic viral antigens capable of eliciting robust humoral and cellular immune responses. However, none have been implemented yet for use as part of a routine swine vaccination program.

While reduction of JE disease and viremia in swine may be possible through pig immunization, it is important to understand that vaccination of domestic pigs cannot be solely relied upon to prevent the risk of human JEV infection and disease to the same extent as direct human immunization (Tsai, 2000). In addition to the rapid turnover of the pig population, the

associated high costs, and the logistics of implementing a new swine vaccination program, alternate vertebrate hosts, such as wild feral pigs or birds, can still amplify the virus and maintain the transmission cycle in the area (Erlanger et al., 2009; Tsai, 2000). For example, based on serological evidence, JEV transmission has continued in Singapore into recent years through the local wild boar and migratory bird population despite the abolishment of domestic pig farming in the early 1990s (Yap et al., 2019). Since the mosquito biting rate is important in the transmission process based on mathematical modeling by Diallo et al. (2018), limiting the potential contact of pigs with JEV-infected mosquitoes may be another method in reducing JE transmission intensity and JE swine disease within pig populations and, thus, subsequent risk for humans. In Australia, the relocation of domestic pigs away from human habitation (approximately 2.5 km for five years) was tested to reduce the contact between the amplifying hosts and mosquito vector, but it did not eliminate JEV risk to humans based on the positive detection of JEV in the local mosquito population (van den Hurk et al., 2008). Movement of domestic pigs of more than 5 km from human habitations may be necessary to see any impact because mean flight distance of mosquitoes, such as *Cx. annulirostris*, is approximately 4.4 km (Bryan et al., 1992; Solomon, 2006). Additionally, JEV may still circulate within vaccinated or previously exposed pig populations (Fan et al., 2010; García-Nicolás et al., 2017; Ladreyt et al., 2019). These findings suggest that swine immunization is helpful to reduce disease in the pig population, but its contribution to reducing transmission risk to humans may not be significant and is probably relatively minimal. The potential high cost and the practicality aspect of administering pig JEV vaccines must also be considered.

Geographic distribution and molecular epidemiology

Japanese encephalitis virus is currently endemic to large parts of Asia and the Pacific region (Centers for Disease Control and Prevention, 2019; World Health Organization, 2019). The large distribution of JEV in Asia covers from southeastern Russia to Japan, Eastern China, Southeast Asia, and India (Platt and Joo, 2006), expanding to Pakistan in the 1980s and Northern Australia through the Torres Strait in the 1990s (Erlanger et al., 2009; van den Hurk et al., 2019). There are two distinct epidemiologic patterns of JE depending on the climate type of the region. The temperate zones, such as Korea, Japan, China, and Nepal, usually experience large epidemics in the summer months (Erlanger et al., 2009). On the other hand, tropical areas, such as Vietnam, Thailand, Malaysia, and Philippines, generally have sporadic cases throughout the year with peak transmission during the rainy months (Erlanger et al., 2009). However, further dispersal of JEV is likely. In Italy, antibodies against JEV and JEV RNA have been detected in local birds sampled from 1996 to 2000 (Preziuso et al., 2018) as well as JEV RNA in field-collected *Cx. pipiens* mosquitoes in 2011 (Ravanini et al., 2012). In addition, the possibility of local transmission of JEV in Africa was reported when JEV genome was detected in a human patient who had not traveled abroad during the 2016 yellow fever outbreak in Angola (Simon-Loriere et al., 2017). The geographic distribution could also potentially change in the future through various forms of introductions or dispersal into new regions including through bird and human migrations, accidental transportation of vectors, and climate change affecting the distribution of vectors or vertebrate hosts (Erlanger et al., 2009). For example, JEV was likely introduced to Northern Australia by wind-blown mosquitoes from Papua New Guinea (Erlanger et al., 2009). As such, the emergence of JEV in new regions is a constant threat. Upon introduction, the presence of

susceptible mammalian or avian hosts and competent vectors in the new regions could establish the virus in the location permanently.

Distribution of genotypes

At the nucleotide level, divergence of 10% in nucleotide sequences has been used to distinguish genotypes of flaviviruses (Beasley et al., 2001). Based on the sequences of the envelope gene, JEV is clustered into five genotypes (I, II, III, IV, and V) (Fan et al., 2013). Genotype I is further classified into two clades (Ia and Ib) (Schuh et al., 2013). In the order of evolution, genotype IV represents the oldest lineage (Solomon et al., 2003). The majority of the natural circulating JEV isolates belong to genotypes Ib and III (Solomon et al., 2003). Except for genotype IV, all are capable of causing human disease (Pan et al., 2011; Solomon et al., 2003). However, there is no clear evidence that there is a clear-cut relationship between genotype and virulence in mouse models (Le Flohic et al., 2013).

The geographic distribution of these genotypes is shown in **Figure 1.3**. Epidemic outbreaks in the temperate zones are usually associated with genotypes Ib and III, whereas genotypes Ia, II, and IV are primarily involved with endemic transmission in the tropical regions (Schuh et al., 2013). Genotype V has recently emerged in 2009 in China and 2010 in South Korea after remaining undetected for almost 60 years since its first isolation in 1952 in Malaysia (Li et al., 2011; Nah et al., 2015; Solomon et al., 2003). Antigenic and genetic variation exists within each genotype, but all groups differ from one another by only 10% to 20% in nucleotide sequences and 2% to 6% in amino acid sequences (Beasley et al., 2004; Burke and Monath, 2001; Nah et al., 2015). Despite this diversity, JEV only has one serotype due to its limited assortment of amino acids (Nah et al., 2015; Tsarev et al., 2000).

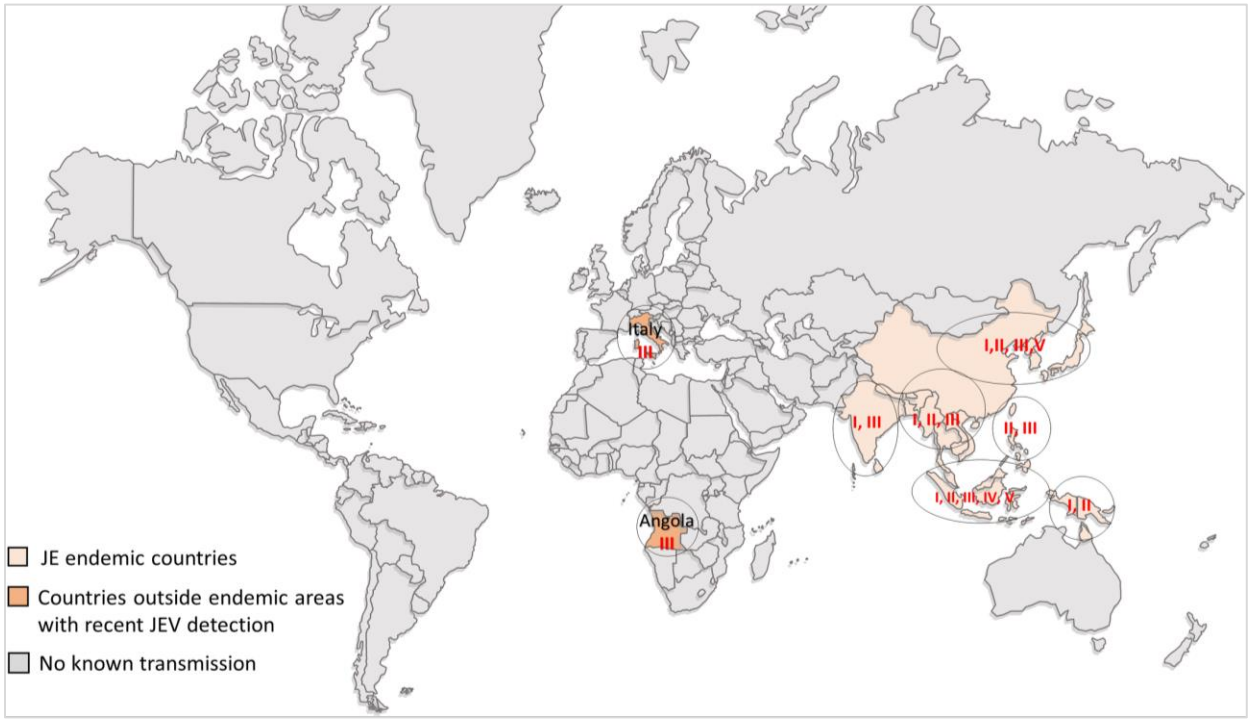

Figure 1.3. Geographic distribution of JEV.
World geographic distribution of JEV genotypes (I to V) with colors representing endemic countries, mostly in Asia, and two countries with recent JEV GIII detection outside its endemic areas. https://doi.org/10.1371/journal.pntd.0008986.g001. (Image taken from Faizah et al. (2021); Published under the Creative Commons Attribution License).

Most human JE outbreaks before the 1970s were associated with genotype III, but a gradual increase in the detection of genotype Ib has been documented in the last few decades (Pan et al., 2011). For example, genotype I replaced genotype III as the dominant lineage in Korea after genotype I was introduced in 1993 (Nah et al., 2015). Genotype I viruses were identified in the majority of samples collected in Japan after 1994, in Thailand after 2000, and in China after 2001 (Morita, 2009; Nah et al., 2015; Pan et al., 2011; Yang et al., 2014b). Following the trend, genotype I displaced genotype III within a year after being introduced in Taiwan in 2008 (Chen et al., 2011; Fan et al., 2013).

Interestingly, Han et al. (2014) found a correlation in an epidemiology study that most

isolates identified as genotype I were collected from mosquitoes and pigs while the majority of human cases were associated with genotype III. However, this observation in host preference does not mean that people are safer in genotype I dominated regions, because acute encephalitis from genotype I has been documented in Japan, India, and China (Pan et al., 2011; Zhang et al., 2011). Instead, while there is no apparent difference in virulence between the two genotypes, minor genetic mutations may have increased the fitness or replication capacity of genotype I viruses in hosts or vectors without altering the pathogenicity or clinical presentation (Nga et al., 2004; Saito et al., 2007; Uchil and Satchidanandam, 2001). Efficient transmission from mosquitoes to pigs or birds and a more efficient overwintering mechanism, which allows the virus to spread and remain year-round in temperate Asia, as additional potential reasons have been proposed (Han et al., 2014; Nemeth et al., 2012; Schuh et al., 2013).

Consequently, several research groups attempted to investigate the possible reasons why genotype Ib displaced genotype III and emerged as the new dominant lineage. In one study, while genotype Ib and III viruses had similar infection rates and reached comparable replication titers in *Cx. tritaeniorhynchus* mosquitoes after oral infection via infected blood meal, genotype Ib viruses generated higher viral titers in cells derived from amplifying hosts than genotype III viruses, especially at elevated temperatures (Fan et al., 2019). Inoculation of pigs, ducklings, and young chickens were also consistent with this finding and demonstrated that genotype Ib infections resulted in earlier and higher viremia when compared to infections by genotype III (Fan et al., 2019). A separate study also demonstrated that experimental infection of a variety of avian species with JEV produced viremia that was relatively higher and of longer duration with genotype Ib infection, suggesting another mechanism of why it has replaced genotype III as the dominant circulating lineage (Nemeth et al., 2012). Mutations of the viral NS2B/NS3 protease may be

responsible for the enhanced infectivity of genotype I (Fan et al., 2019). At the same time, other studies have demonstrated no difference between genotype I and III in the magnitude or duration of viremia in chicks, young ducklings (Cleton et al., 2014), and pigs (Xiao et al., 2018a). Understanding the pathogenesis of JEV may provide additional insight into the major drivers of genotype emergence.

Molecular biology and pathogenesis

Virus structure and genome

Japanese encephalitis virus is an enveloped and spherical virus with an icosahedral geometry and is approximately 40 nm in diameter, as displayed in **Figure 1.4** (Tsai, 1990; Wang et al., 2017b). The surface of the virus is covered by 180 heterodimers of envelope (E) and membrane (M) glycoproteins lying antiparallel to each other (Wang et al., 2017b). Encapsidated under this envelope is a single-stranded, positive-sensed, 11 kb RNA genome flanked by 5′ and 3′ untranslated regions in a polyhedral capsid (Li et al., 2017b; Platt and Joo, 2006). As shown in **Figure 1.5**, the genome consists of one single open reading frame that is translated into a single polyprotein that is cleaved post-transcriptionally by viral and host enzymes into ten important viral proteins. There are three structural proteins (C, prM/M, and E) involved in viral maturation, attachment, and entry and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) that are critical for viral RNA replication, translation, and immune escape (Li et al., 2017b; Schuh et al., 2013; Yang et al., 2014a). The major functions of each viral protein are reviewed in **Table 1.2**.

Members of the JE serocomplex express an additional form of NS1 designated as NS1′ protein (Rastogi et al., 2016), a -1 programmed ribosomal frameshift product that results in the

addition of 52 amino acids to the C-terminus of NS1 (Melian et al., 2010). It is expressed in approximately 30% to 50% of translational events (Melian et al., 2014; Melian et al., 2010; Young et al., 2015) and likely shares NS1 functions (Satchidanandam et al., 2006; Takamatsu et al., 2014; Young et al., 2015) in addition to having unique immune evasive functions (Zhou et al., 2018; Zhou et al., 2020).

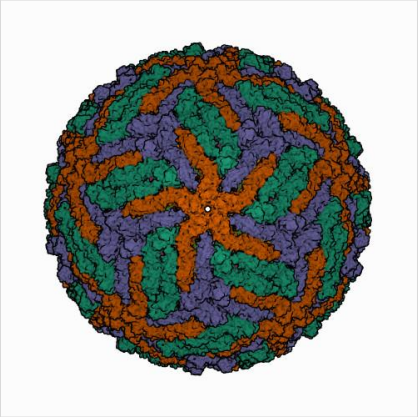

Figure 1.4. Structure of Japanese encephalitis virus.
Image from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB [rcsb.org]) of PDB ID 5WSN (Wang et al., 2017b). Data files contained in the archive are free of all copyright restrictions.

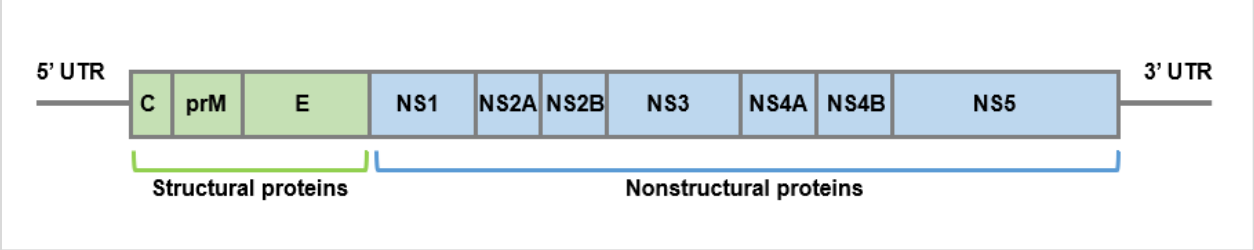

Figure 1.5. Schematic diagram of the JEV genome.

Table 1.2. Summary of the major functions of JEV proteins.

Protein type	Viral proteins	Major functions	References
Structural proteins	Capsid (C)	▪ Binds with viral RNA to form the ribonucleoprotein complex	(Lu et al., 2017)
	Pre-membrane/ Membrane (PrM/M)	▪ Pr peptide prevents premature viral fusion by obscuring the fusion loop on the E protein of immature virions ▪ M is a chaperone for the correct folding of E protein	(Carbaugh and Lazear, 2020)
	Envelope (E)	▪ Role in viral attachment, membrane fusion, and neuroinvasion ▪ Domain I – contains the N-terminus signal peptide sequence to direct the endoplasmic reticulum (ER) membrane topology of the flavivirus structural polyprotein region; structurally the central domain of E protein ▪ Domain II – contains the dimerization domain and hydrophobic fusion loops necessary for virus-host membrane fusion to release the viral genome into the host cytoplasm ▪ Domain III – mediates virus attachment and induces potent neutralizing antibodies	(Carbaugh and Lazear, 2020; Fan et al., 2012; Robbiani et al., 2017; Wahala and Silva, 2011; Zhang et al., 2017)
Nonstructural proteins	Nonstructural protein 1 (NS1)	▪ ER-lumen component of the virus replication complex ▪ Frequently used as a diagnostic marker of infection due to its highly immunogenic and conserved nature among flaviviruses ▪ Triggers endothelial hyperpermeation ▪ Immune evasive functions: e.g. inhibits complement activation, inhibits TLR3, and suppresses ROS and JAK-STAT pathways in the mosquito midgut	(Chung et al., 2006; Liu et al., 2016; Puerta-Guardo et al., 2019; Wang et al., 2019; Wilson et al., 2008)
	NS1′	▪ Can substitute NS1 in the virus replication complex ▪ Associated with neuroinvasion and neurovirulence in mice ▪ Immune evasive functions: e.g. antagonizes interferon (IFN)-β production by targeting MAVS	(Satchidanandam et al., 2006; Takamatsu et al., 2014; Ye et al., 2012; Young et al., 2015; Zhou et al., 2018; Zhou et al., 2020)
	NS2A	▪ Transmembrane component of the virus replication complex	(Li et al., 2017b; Liu et al., 2006; Qiu et al., 2020)

		▪ Generates virus-induced membranes for virus assembly in the ER ▪ Immune evasive functions: e.g. suppresses RNA interference and suppresses IFN-β transcription	
	NS2B	▪ Transmembrane component of the virus replication complex ▪ Forms the viral protease with NS3 as an essential co-factor	(Lu et al., 2017; Shah et al., 2018)
	NS3	▪ Cytoplasmic component of the virus replication complex ▪ N-terminal serine protease with NS2B ▪ C-terminal helicase and nucleoside 5'-triphosphatase	(Lu et al., 2017; Shah et al., 2018)
	NS4A	▪ Transmembrane component of the virus replication complex ▪ Remodels ER membranes to create the replication site ▪ Immune evasive functions: e.g. inhibits dsRNA-activated protein kinase R	(Fan et al., 2016; Shah et al., 2018)
	NS4B	▪ Transmembrane component of the virus replication complex ▪ Immune evasive functions: e.g. interferes with type I IFN signaling, RNAi, formation of stress granules, and unfolded protein response	(Zmurko et al., 2015)
	NS5	▪ Cytoplasmic component of the virus replication complex ▪ Largest protein ▪ RNA methyltransferase and RNA-dependent RNA polymerase ▪ Immune evasive functions: e.g. inhibits IFN-β-induced apoptosis, suppresses type I IFN production, interferes with dsRNA-induced nuclear translocation of IRF3 and NF-kB, and blocks IFN-stimulated JAK-STAT signaling	(Li et al., 2017b; Lin et al., 2006; Shah et al., 2018; Weng et al., 2018; Ye et al., 2017)

Virus replication

Upon recognition and attachment of specific receptors, such as heparin sulfate, DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin) or CD29, and mannose receptor, on the cell surface, JEV is internalized via clathrin-mediated endocytosis (Das

et al., 2009; Kaufmann and Rossmann, 2011; Zhao et al., 2020). Following endocytosis, the acidic environment of the endosome causes conformational changes of the E proteins from dimers to trimers, which exposes its hydrophobic fusion loops to initiate the pH-mediated fusion of the viral and endosomal membranes (Kaufmann and Rossmann, 2011; Li et al., 2017b; Stiasny and Heinz, 2006). This results in the release of the viral genome into the cytoplasm, leading to its translation into polyproteins that are post-transcriptionally cleaved into the structural and nonstructural viral proteins and to initiate the synthesis of viral RNA (Yang et al., 2014a). Virus replication and assembly then occurs in the endoplasmic reticulum to produce immature, fusion-incompetent viral particles that undergo pH- and furin-dependent maturation during their transit through the secretory pathway via the *trans*-Golgi network to the cell surface (Kaufmann and Rossmann, 2011). The newly assembled immature virions have a rough spikey surface composed of E trimers associated with prM (Wang et al., 2017b). The acidic environment in the *trans*-Golgi network causes conformational changes in the E protein and allows the glycosylated N-terminal pr fragment to be cleaved by the cellular endoproteinase furin from the prM protein (Kaufmann and Rossmann, 2011). The pr fragment remains associated with the virion until they are shed during exocytosis and release of the virus, resulting in the membrane-anchored M proteins to reorganize with the E proteins into dimers and into their mature infectious metastable structural state (Kaufmann and Rossmann, 2011).

Pathogenesis of JEV in the vertebrate host

Human infections

The primary insult starts with the injection of JEV into the skin through the bite of an infected mosquito, infecting a variety of skin-resident cells including keratinocytes, Langerhans

cells, stromal cells, dendritic cells, and macrophages (Fong et al., 2018). The local infection then triggers the recruitment of additional susceptible immune cells to the site, leading to the migration of infected antigen presenting cells, such as Langerhans and dendritic cells, to local draining lymph nodes where more susceptible cells are populated (Johnston et al., 2000; Wang et al., 2017a). Consequently, the virus infection spreads from the regional lymph nodes through the lymphatics to blood, leading to viremia. As a highly neurotropic virus, it ultimately reaches the central nervous system after additional nonspecific replication in tissues such as skeletal muscle and liver (Platt and Joo, 2006). As such, the permissiveness of myeloid dendritic cells and macrophages to JEV infection is closely associated with the degree of neuroinvasion (Wang and Deubel, 2011).

However, the exact mechanisms of neuroinvasion is unclear. One of the proposed routes of neuroinvasion by JEV is the increase of permeability and subsequent breakdown of the blood-brain barrier by the production of inflammatory cytokines (Mathur et al., 1992; Monath et al., 1983; Myint et al., 2014; Nagata et al., 2015). Mathur et al. (1992) demonstrated that a JEV-induced cytokine, splenic macrophage-derived neutrophil chemotactic factor, caused dose-dependent vascular leakage of the blood-brain barrier, which also correlated to clinical sickness and virus titer in the brain, without inducing morphological damage to the endothelium in mice. Japanese encephalitis virus could also potentially reach the brain through other routes including via blood (Myint et al., 1999; Redant et al., 2020; Yamada et al., 2004), infected endothelial cells (Myint et al., 1999), infected lymphocytes (Mathur et al., 1988; Myint et al., 1999; Nagata et al., 2015; Yamada et al., 2004), transcytosis (Clark et al., 2012; Liou and Hsu, 1998), and retrograde axonal transport through the olfactory pathway (Clark et al., 2012; Yamada et al., 2009). Ultimately, JEV most likely reaches the central nervous system through a combination of these proposed routes, followed by viral dissemination in the brain through the extracellular space and/or

by direct intercellular spread (Ayala-Nunez and Gaudin, 2020; Tsai, 1990).

Once within the brain, JEV causes neuronal cell death via direct killing through viral replication and induction of apoptosis in addition to indirect damage by inducing a massive inflammatory response (Ghosh and Basu, 2009). Neuronal death then activates microglia and astrocytes to release pro-inflammatory cytokines, such as tumor necrosis factor-α, reactive oxygen species, and interleukin-6, that causes further tissue damage and promote massive leukocyte migration and infiltration into the brain (Ghosh and Basu, 2009; Ghoshal et al., 2007; Myint et al., 2014). Consequently, more subsequent neuronal death stimulates the inflammatory cycle to continue inappropriately (Ghosh and Basu, 2009). Uncontrolled inflammatory cytokine production in the brain is a characteristic immunopathology of JE in humans (Ghosh and Basu, 2009). The observed persistent neurologic deficits in survivors are most likely consequences of the profound destruction of neurons during the acute infection involving virus-induced neuronal death, host inflammatory responses, and autoimmunity to previously hidden neural antigens (Clark et al., 2012; Desai et al., 1994). Neuronal necrosis, vascular disruption, and moderate to severe inflammation in the brain, especially in the gray matter, are the most prominent histopathological changes (Clark et al., 2012).

Swine infections

In contrast to the extensive increase of pro-inflammatory cytokines observed in humans, primates, and mice, JEV replication in the brain of pigs is mostly efficiently suppressed, predominantly by type I interferon-independent activation of OAS1 (2'-5'-oligoadenylate synthetase 1) expression and increased interferon-gamma activity (Redant et al., 2020). In other words, the acute inflammatory responses in the brain are differently regulated in humans and pigs.

In the study by Redant et al. (2020), no marked increases in mRNA expression of pro- and anti-inflammatory cytokine genes were detected in JEV-infected porcine brain tissues. Expression profiles of interferon-α and -β were also unchanged, but the antiviral OAS1 gene was moderately upregulated in the brain tissues of JEV-infected pigs, resulting in the activation of RNase L, which has a critical role in the degradation of viral RNA and suppression of viral protein synthesis (Redant et al., 2020; Silverman, 2007). This tight regulation or inhibition of the pro-inflammatory response may partly explain why JEV infection in pigs is mostly mildly clinical without the induction of significant neurological signs (Redant et al., 2020). Additionally, JEV does not induce a systemic inflammatory cytokine response in infected pigs, supporting a rapid control of virus replication (Ricklin et al., 2016b). Production and levels of inflammatory cytokines, including interferon-α, interleukin-1β, tumor necrosis factor-α, and interleukin-6, remained unchanged and were indistinguishable between the needle-infected and non-infected control pigs (Ricklin et al., 2016b). These observations could be partly explained by a study conducted by Espada-Murao and Morita (2011) that demonstrated that the cytosolic exposure of double-stranded JEV RNA during virus replication was significantly delayed in interferon-competent porcine cells (i.e. porcine kidney PS, PK, and ESK cells) compared to primate cells (i.e. rhesus monkey kidney LLC-MK2 cells), correlating with the delayed detection, delayed interferon response, and subsequent enhanced viral dissemination.

In pigs, the different modes of JEV infection (i.e. needle [intravenous and intradermal] vs. oronasal vs. direct contact) result in similar pathologic outcomes and immune responses (Redant et al., 2020; Ricklin et al., 2016a). Peak viremia is reached at 2 to 3 days post-infection with evidence of viral tissue dissemination and neuroinvasion at that time point (Redant et al., 2020; Ricklin et al., 2016a; Ricklin et al., 2016b; Young et al., 2020). Lymphoid tissues typically only

demonstrate slight follicular hyperplasia (Ricklin et al., 2016a). In young pigs, central nervous system lesions typical of nonsuppurative encephalitis, consisting of perivascular cuffing with lymphocytes, multifocal gliosis, and neural degeneration and necrosis are most prominent at 5 days post-infection (Fujisaki, 1975; Ricklin et al., 2016a; Ricklin et al., 2016b; Yamada et al., 2009). In pregnant sows, virus can reach the fetus by day 7 post-infection (Platt and Joo, 2006). If the sow is infected before 60 to 70 days of gestation, sequential infection of litter similar to porcine parvovirus may occur, leading to a mixture of normal, weak, stillborn, and/or mummified fetuses of different stages (Platt and Joo, 2006; Shimizu et al., 1954; Sugimori et al., 1975). Stillborn piglets commonly demonstrate pathological lesions in the brain such as hydranencephaly, diffuse edema, congestion, dilation of ventricular spaces, and neuronal degeneration along with congested lymph nodes (Desingu et al., 2016). Subcutaneous edema, hydrothorax, cerebellar hypoplasia, and spinal hypomyelinogenesis can also be observed (Burns, 1950; Morimoto, 1969). In addition, viral oronasal sheddings capable of infecting immunologically-naïve pigs by direct nose-to-nose contact can be detected within the first week post-infection in pigs under laboratory settings (García-Nicolás et al., 2017; Redant et al., 2020; Ricklin et al., 2016a). The shedding source is most likely a combination of virus release from the nasal epithelium (García-Nicolás et al., 2018) and indirect reflection of blood as oral mucosal transudate (Thompson and Benjamin, 2019), but not necessarily from the tonsil (García-Nicolás et al., 2017). The majority of these pathogenesis studies have been performed in Europe or Asia with their local domestic commercial pigs, primarily with genotype III, through intravenous challenge, and without the involvement of mosquito saliva, which is an important component in JEV transmission in nature that is known to modulate arboviral infection and disease.

Effect of mosquito saliva in JEV pathogenesis

Infectious viruses are not the only thing being inoculated into the vertebrate host when an infected mosquito feeds on the host. Mosquitoes inject approximately 30% to 40% of their active salivary factors or the equivalent of 0.3 to 0.4 salivary gland pairs/ml in the skin during a feed (Schneider and Higgs, 2008). Mosquito saliva is a complex concoction of more than 100 proteins, majority of which have functions yet to be determined (Thangamani and Wikel, 2009; Vogt et al., 2018). An estimate of around 140 putative secreted proteins are found in the mosquito saliva that could modulate the host immune responses and consequently impact the disease pathogenesis of the arbovirus in the vertebrate host (Thangamani and Wikel, 2009). Exogenous microRNAs are also secreted in the mosquito saliva that can potentially alter the efficiency of virus replication (Maharaj et al., 2015). Based on several mouse studies, it is becoming increasingly recognized that mosquito salivary components can antagonize the host antiviral immune response and subsequently cause enhancement of virus replication and disease severity. Reported effects of mosquito saliva that may facilitate and promote virus replication in the host include induction of anti-inflammatory cytokines (Schneider et al., 2004; Vogt et al., 2018), inappropriate polarization from a Th1 to Th2 immune response (Schneider et al., 2004; Thangamani et al., 2010), and suppression of the host innate immune response (Schneider and Higgs, 2008; Thangamani et al., 2010). In addition, mosquito salivary components can promote extensive cutaneous edema which leads to prolonged retention of virus at the inoculation site (Pingen et al., 2016), enhance recruitment of inflammatory cells to the site as additional cellular targets of virus replication (Conway et al., 2014b), and disrupt endothelial barriers which may facilitate virus dissemination (Fong et al., 2018; Schneider and Higgs, 2008). Altogether, these modulatory effects of the mosquito saliva in the host may help explain why enhanced virus replication, dissemination, and

clinical disease has been observed in many challenge studies of several arboviruses including La Crosse (Osorio et al., 1996), Cache Valley (Edwards et al., 1998), and West Nile viruses (Moser et al., 2016; Styer et al., 2006; Styer et al., 2011). Brief summaries of these studies are provided in **Table 1.3**.

Nevertheless, the significance of mosquitoes on the pathogenesis of arboviruses requires further investigation. The reported effects of mosquito saliva through the bites of infected mosquitoes and/or its equivalent of co-injecting virus and salivary gland extract has not been universal or consistent across all studies. For example, no demonstrable changes were observed in the infection or pathology outcomes by mosquito bite infection of chickens with Western equine encephalitis (Reisen et al., 2000) or West Nile viruses (Langevin et al., 2001) and of hamsters with West Nile virus (Sbrana et al., 2005) compared to virus-only needle inoculations. This raises the question of why do not all hosts develop more severe arbovirus infections by administering mosquito saliva in these experimental needle challenges? Other important factors may be at play which contribute to the overall impact of the mosquito in the pathogenesis of arboviral disease in the vertebrate host including the dose ratio of mosquito saliva to virus (Le Coupanec et al., 2013; Moser et al., 2016), the age of the susceptible host (Styer et al., 2006), and the source or species of mosquitoes (Le Coupanec et al., 2013; Wanasen et al., 2004).

Table 1.3. Examples of studies demonstrating the enhancement of arbovirus infection and disease by the addition of mosquito saliva.

Virus	Results	Reference
La Crosse virus (LACV)	White-tailed deer and chipmunks developed higher and longer viremia when infected with bites from LACV-infected *Aedes triseriatus* mosquitoes compared to virus-only needle challenge	(Osorio et al., 1996)
Cache Valley virus (CVV)	Outbred ICR mice resistant to virus-only needle challenge developed viremia and antibody response when CVV was injected into feeding sites of non-infected mosquitoes (*Aedes triseriatus*, *Aedes aegypti*, and *Culex pipiens*)	(Edwards et al., 1998)
West Nile virus (WNV)	Dose-dependent enhancement of viremia in C57BL/6 mice was observed with the number of *Culex tarsalis* mosquitoes spot-feeding at the inoculation site or with the amount of salivary gland extract administered with the needle WNV challenge	(Moser et al., 2016)
	Higher viral titers in serum, oral swabs, and cloacal swabs were detected in chicks earlier when infected by bites from WNV-infected *Culex pipiens* mosquitoes compared to virus-only needle challenge	(Styer et al., 2006)
	C57BL/6 mice infected by bites from WNV-infected *Culex tarsalis* mosquitoes developed higher viremia, higher tissue titers, and faster neuroinvasion compared to virus-only needle challenge	(Styer et al., 2011)

Studies investigating the impact of mosquito saliva in the disease pathogenesis of JEV has been limited. Furthermore, the results that has been published thus far are inconsistent with each other. In the mouse study by de Wispelaere et al. (2017), infection dynamics and outcomes were indistinguishable when challenge was conducted by virus-only needle inoculation, injection of

JEV-infected mosquito saliva, or co-administration of JEV and salivary gland extract collected from European populations of *Cx. pipiens* or *Aedes albopictus*. However, the population source of the mosquito species could be a factor in these observations, because specific mosquito species from different geographic locations can be genetically distinct (Fonseca et al., 2004). In contrast, 2-day-old ducklings infected by bites from JEV-infected *Cx. pipiens* resulted in 30% mortality rate from viral encephalitis by 3 days post-infection and displayed neurologic signs of opistothonos (Di et al., 2020), which were not observed with subcutaneous injection challenge of JEV (Xiao et al., 2018b). These two model systems established thus far provide inconsistent results. How mosquito saliva may truly affect the development of disease in humans or its common immunology animal model, the pig, remains undetermined.

Host adaptive immune response to JEV infection

Characterizing the host immune response to virus infection provides important information on how the virus establishes its infection in the host. Neutralizing antibody response to JEV infection can be detected as early as 5 days post-infection in infected pigs (Ricklin et al., 2016b). Early and high neutralizing antibody responses may be crucial for preventing viral neuroinvasion and host fatality (Wang and Deubel, 2011). Protection by neutralizing antibodies to JEV is well established such that neutralizing antibody titers of $\geq$ 1:10 are accepted as evidence of protection and seroconversion (Fujisaki, 1975; Tsai, 1990; Turtle et al., 2016). Cellular immunity to JEV is less well studied, but its induction of strong and persistent memory T cell responses is one of the important hallmarks for successful vaccination and therefore possibly as protection marker (Salerno-Gonçalves and Sztein, 2006). However, no simple correlation of T cell response exists for protection against JEV (Robinson and Amara, 2005). Nonetheless, several studies have

demonstrated the importance of cell mediated immunity to induce an effective antiviral response against JEV infection. For example, many studies have demonstrated that the adaptive transfer of JEV-primed T cells was capable of protecting mice from lethal JEV challenge (Larena et al., 2013; Mathur et al., 1983; Murali-Krishna et al., 1996; Wang et al., 2020) in addition to mediating cross-protection against Zika virus challenge better than the passive transfer of JEV immune sera to protect against Zika virus (Wang et al., 2020). In clinical cases, the type of CD4+ T cell response has been associated with disease outcome of JE patients such that the higher quality and polyfunctional cellular responses were closely correlated with complete recovery from JE (Turtle et al., 2016). Additionally, interferon-gamma, which is primarily produced by activated T cells, has been identified as a critical component in viral clearance and patient recovery by suppressing virus replication in the central nervous system (Larena et al., 2013; Zia et al., 2017).

In summary, the activation of the humoral and cellular immune responses in the infected host are necessary to target, neutralize, and eliminate the virus from the body. Although very uncommon, JEV can sometimes successfully evade and remain hidden from the host immunity and cause persistent infections.

Persistent infection of JEV

While JEV infections are primarily described as acute infection and disease, recrudescence of symptoms and persistent JE infection can be observed in humans (Tsai, 1990). Persistent and latent infections of JEV has been previously reported in several cell cultures such as murine neuroblastoma (Chen et al., 1996) and murine microglial cells (Thongtan et al., 2010), mice models (Mathur et al., 1982; Mathur et al., 1986a, b; Mathur et al., 1989; Thongtan et al., 2010), and in T-lymphocytes (Sharma et al., 1991) and nervous system (Ravi et al., 1993) of human patients.

Reactivation of JEV has been possible in some of these studies through immunosuppressant drugs (Mathur et al., 1986b), pregnancy (Mathur et al., 1982; Mathur et al., 1986a, b), and allogeneic (Mathur et al., 1986a) or xenogeneic (Sharma et al., 1991) stimulation.

Pigs present a good model to investigate persistent JEV infection, especially in immune-related cell types. Persistence of JEV in tissues long after the acute phase of infection is a recent novel observation in pigs (Ricklin et al., 2016a). Persistence of JEV in the tonsil of infected pigs have been detected for up to 46 days post-infection based on RT-qPCR, suggesting that the virus may somehow be hidden from the host immune response (García-Nicolás et al., 2017). JEV RNA was also detectable in the brain at 21 day post-infection after oronasal challenge (Ricklin et al., 2016a). These findings warrant further investigation to determine if pigs, in addition to being efficient amplifiers, can also function as "silent" carriers of JEV, capable of re-shedding and/or developing recurrent infections after initial exposure. Such knowledge can help better define the roles of pigs as an amplifying host in nature.

Justification for research

The overarching goal of this dissertation is to determine the susceptibility of North American pigs to JEV infection. It is undoubtedly clear that JEV is an important pathogen that requires continued studies and further research to control the disease. The potential introduction and subsequent outbreaks of JEV in new territories is, thus, a significant concern for both public and animal health worldwide. Although JEV is currently only reported to be endemic to the Asia-Pacific region (World Health Organization, 2019), there is available evidence that highlight the possibility of this exotic arbovirus becoming established in North America after a dispersal or introduction event.

Competent mosquito species are present in North America

Based on experimental infections of laboratory colonies with JEV genotype III strain, several western North American mosquito species have been identified that are competent transmitters of JEV (Reeves and Hammon, 1946). The potential vector species included *Cx. pipiens, Cx. quinquefasciatus, Cx. tarsalis, Ae. nigromaculis, Ae. dorsalis, Culiseta incidens*, and *Culiseta inornata*, most of which are also known vectors of encephalitic arboviruses already endemic in the region such as Western equine encephalitis and St. Louis encephalitis viruses (Reeves and Hammon, 1946). In a more recent study, susceptibility following oral challenge with genotypes I and III viruses was demonstrated for *Cx. quinquefasciatus* collected from Valdosta, Georgia (Huang et al., 2016a; Huang et al., 2015). Transmission of JEV was possible by 14 days post-infection based on the detection of viral RNA in the mosquito saliva, suggesting that *Cx. quinquefasciatus* is a competent vector of JEV (Huang et al., 2016a; Huang et al., 2015). These mosquitoes are opportunistic blood feeders and can be primarily anthrophilic in urban settings (Farajollahi et al., 2011; Lourenço-de-Oliveira et al., 2018), thereby capable of having a role in enzootic or epizootic JEV cycles.

Since evidence suggests that several North American *Culex* species mosquitoes are competent for JEV transmission, their geographic distribution would provide the basis for mapping of high risk areas. As shown in **Figure 1.6**, *Cx. pipiens* can be primarily found in the urban areas in the north, *Cx. quinquefasciatus* in the sub-urban temperate and tropical regions in the south, and *Cx. tarsalis* in areas west of the east coast (Evans et al., 2017; Farajollahi et al., 2011). Additionally, they are the most common mosquitoes in urban areas and altogether cover the entire country in geographic distribution (Diaz-Badillo et al., 2011). This could be one of the reasons

West Nile virus quickly spread nationwide after its initial introduction in 1999 in New York, because these mosquitoes are also considered the primary vectors of WNV in North America (Diaz-Badillo et al., 2011). Since West Nile virus is a close relative to JEV with similar enzootic cycle characteristics, JEV could potentially exploit the same mechanisms and become established in the region.

Figure 1.6. Distribution maps of *Culex pipiens, Culex quinquefasciatus,* and *Culex tarsalis* in the continental U.S.

(Image modified from (Evans et al., 2017); Published under the Creative Commons Attribution License).

Susceptible North American avian species have been identified

Hematophagous arthropod vectors are just one component of arboviral transmission cycles. Without the presence of amplifying vertebrate hosts, transmission cycles of most arboviruses in a region cannot be established. As such, the susceptibility of several species of birds found in North America to JEV infection have been determined. Researchers from Colorado demonstrated that these birds can develop viremic profiles capable of supporting JEV transmission as amplifying hosts (Nemeth et al., 2012). In addition to water-wading birds that JEV is usually closely associated with such as egrets, other avian species including rock pidgeons (*Columba livia*), house finches

(*Carpodacus mexicanus*), and common grackles (*Quiscalus quiscula*) were capable of developing high and prolonged viremia after subcutaneous inoculation with JEV (Nemeth et al., 2012). While no clinical disease ever developed, viremia reached approximately as high as 10^4 $TCID_{50}$/ml and lasted up to 7 days post-infection in some species (Nemeth et al., 2012). This study also helped identify the important avian species to focus for optimal JEV surveillance.

The role of North American domestic and feral pigs in JEV transmission

Pigs are another important amplifying host of JEV, but it has yet to be investigated whether the pig populations from North America are susceptible to JEV and could serve as amplifying hosts. This critical but missing knowledge is essential to properly assess the potential for JEV to establish local enzootic transmission cycles in North America.

Although domestic pigs from different regions are still classified under the same scientific name *Sus scrofa*, variation in disease susceptibility based on locality or breed has been observed in both experimental and natural conditions. In particular, breed is recognized as an important factor that determines resistance or susceptibility in pigs to several viral infections (Blacksell et al., 2006; Meng et al., 2018; Mujibi et al., 2018; Opriessnig et al., 2006; Xing et al., 2014). For example, the local indigenous pig breeds or those with higher local breed ancestry were associated with being more robust against classical swine fever (Blacksell et al., 2006) or African swine fever virus (Mujibi et al., 2018) infections. Breed-dependent differences in susceptibility in terms of severity of clinical disease have been demonstrated for porcine reproductive and respiratory syndrome virus (Meng et al., 2018; Xing et al., 2014) and porcine circovirus type 2 virus infection (Opriessnig et al., 2006). Additionally, the same breed category can be genetically different based on the place of origin. In a study of pig genetic diversity, Landrace pigs from different countries

(Sweden and Germany) formed distinct separate clusters as two different breeds based on a panel of microsatellite markers recommended by the Food and Agriculture Organization of the United Nations for diversity analysis (Laval et al., 2000). With JEV, breed or strain differences in the impact of JE reproductive disease has been suggested in pigs (Lindahl et al., 2012; Sugawara et al., 1974) and demonstrated in mice (Fujisaki et al., 1976). These findings suggest that it is important to directly assess the susceptibility of animals from specific regions to the virus of interest to obtain the most correct information.

In addition to characterizing the susceptibility profile of the domestic pigs from North America to JEV, the potential role of the North American feral swine must also be investigated because they are presumed to be important in the endemic areas. Since the first introduction of pigs into the United States in the 13th century, subsequent accidental escapes, and deliberate release for game (McCann et al., 2018), there is now a significant wild pig population in North America. Currently, there is an estimate of over 6 million feral wild pigs roaming in the United States in at least 35 states and their overpopulation has created significant cost of approximately $1.5 billion U.S. dollars in damages and control measures associated with agriculture, property, and disease transmission (Animal and Plant Health Inspection Service, 2020; McCann et al., 2018). Their explosive population and distribution continue to expand northward (McCann et al., 2018; Ruiz-Fons, 2017). Wild swine is still considered *Sus scrofa*, but it is widely established that they are genetically distinct from domestic pigs so how a virus establishes its infection in one or the other host cannot be rationally extrapolated. Several different panels of microsatellite markers developed to aid in the selection of economically important traits, such as growth rate, fecundity, and disease resistance, can be used to distinguish wild boar from domestic pigs (Conyers et al., 2012; Costa et al., 2012; Lowden et al., 2002). Although limited, evidence also exists showing that wild pigs can

have different susceptibility profiles or disease outcome from viral infections compared to domestic pigs. For example, Brugh et al. (1964) demonstrated that wild swine had shorter prodromal periods and faster rates to death from classical swine fever virus infection compared to domestic pigs. Genetics, parasitic infections, and inoculum dosage versus pig size were identified as potential factors that contribute to the observed differences (Brugh et al., 1964). With JEV, it is known that feral pigs are susceptible and exposed in nature based on serological data, but their infection outcomes including clinical and pathological changes are unknown at this time.

Hypothesis and specific aims

Despite the extensive studies on JEV since its initial isolation in 1924 (Erlanger et al., 2009; Tsai, 1990), much of its pathogenesis, especially in North American domestic and feral pigs, still requires further investigation. The objective of this dissertation was to address this important research gap and determine the susceptibility profile and pathogenesis of JEV in North American pigs. The central hypothesis for this study is that North American domestic and feral pigs are susceptible to JEV and can potentially support its transmission.

The following three specific aims were pursued to test the central hypothesis:

Specific aim 1. To determine the susceptibility of North American domestic pigs to JEV after intravenous challenge. Three-week-old domestic piglets were intravenously challenged with JEV genotype Ib JE-91 strain to characterize their clinical signs, viremia kinetics, viral shedding profiles, and other pathological changes. The invasive intravenous challenge route was selected to induce the artificial viremia needed for viral tissue dissemination and evaluate if pathology or disease will establish in the animal. It was also selected because other JEV challenge studies used the similar approach for disease characterization (Ricklin et al., 2016b; Yamada et al.,

2004). This will allow the comparison of susceptibility and infection outcomes between domestic pigs in North America and other regions. Results are reported in Chapter 3 of this dissertation.

Specific aim 2. To evaluate the effect of mosquito saliva in JEV pathogenesis in North American domestic pigs. The natural route of transmission was mimicked more closely via intradermal inoculation of JEV supplemented with mosquito salivary gland extract. Three-week-old domestic piglets were challenge using this established method of inoculation to investigate the effects of mosquito saliva in the disease pathogenesis of JEV. Data from this study is presented in Chapter 4.

Specific aim 3. To establish a North American feral swine model for JEV pathogenesis. To determine the susceptibility of feral pigs to JEV infection, the Sinclair miniature research swine was selected as a feral pig representative of North America. Three-week-old piglets from this breed was intradermally challenged with JEV to characterize the pathogenesis and compare the infection outcomes to those observed in infected domestic pigs. Chapter 5 contains the results of this experiment.

Together, the findings from these studies will provide a better understanding of how JEV behaves in its enzootic hosts – the domestic and feral pigs. The knowledge generated from the proposed studies will make a positive impact on public health and the security of U.S. agriculture and livestock. The animal models generated through this dissertation work will provide invaluable aid in the development and implementation of effective countermeasures against this disease and in the research of efficacious therapeutic or prophylactic treatments.

Chapter 2 - Materials and methods

Introduction

This chapter provides the collective research approaches, technical methods, and materials used to conduct the dissertation studies since common methods were used for many of the experiments. Details of the publication citation or status of the materials presented in this chapter can be found in their respective sections (i.e. Chapters 3, 4, and 5).

Virus and cell lines

Japanese encephalitis virus strain JE-91, originally isolated in 1991 from mosquitoes collected in Korea (Huang et al., 2016a; Schuh et al., 2010), was used as a representative for genotype Ib for all virus infections and challenges. The nucleotide and amino acid sequences of its envelope protein has been previously determined (GenBank access number: GQ415355). Prior to the experiments, the virus was passaged once in African green monkey (*Cercopithecus aethiops*) kidney epithelial Vero76 cells and once in *Aedes albopictus* C6/36 cells. The virus stock was stored at -80 °C until use.

Both Vero76 and C6/36 cell lines were maintained in Leibovitz (L-15) media supplemented with 10% heat-treated fetal bovine serum (FBS), 10% tryptose phosphate broth (TPB), 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 2 mM of L-glutamine mixture, as previously described (Huang et al., 2016a). Vero76 and C6/36 cells were maintained at 37 °C and 28 °C, respectively, without CO_2. Vero76 cells were primarily used for the titration of virus stock or experimental samples and for serology work. C6/36 cells were used for virus propagation.

Preparation of mosquito salivary gland extract

Salivary glands were dissected from a colony of *Cx. quinquefasciatus* (F>30) originally obtained from Vero Beach, FL (Ayers et al., 2018; Huang et al., 2016b). The colony was tested negative for known flaviviruses using the pan-flavivirus EMF1-VD8 primer set (Cook et al., 2018). Seven- to ten-day-old female mosquitoes were surface sterilized with 70% ethanol and dissected in phosphate buffer saline (PBS) to obtain their salivary glands. Fifty pairs of salivary glands were placed in approximately 1 ml of PBS, sonicated, and centrifuged at 13,000 rpm for 10 minutes at 4 °C to release proteins and pellet cellular debris (Schneider et al., 2010). To obtain the salivary gland extract (SGE), the supernatant was collected and stored at -80 °C until the challenge experiment for Aim 2 study.

Animal experiments and study design

The following experimental procedures and animal use were approved by the Kansas State University (K-State) Institutional Animal Care and Use Committee (IACUC). All methods were carried out in accordance with the approved protocol and relevant regulations. All animal work were conducted in biosafety level 3 agriculture (BSL3-Ag) conditions at the Biosecurity Research Institute at K-State, Manhattan, KS. Animals were allowed to acclimate for five days in the BSL3-Ag housing prior to the start of the experiments.

Aim 1 research design

The objective for Aim 1 was to characterize the susceptibility of North American domestic pigs through the invasive challenge of intravenous inoculation. Fourteen three-week-old U.S.

commercial pigs (white-line crossbreed) were inoculated intravenously via the right jugular vein with either 1 ml of JE-91 JEV strain at 10^7 $TCID_{50}$/ml (n = 10) or 1 ml of sterile saline (n = 4). Challenge and control pigs were kept in separate pens to avoid the non-vector transmission described by Ricklin et al. (2016a). To characterize the acute and convalescent stages of infection, groups of seven pigs (five infected and two control pigs) were sacrificed at days 3 and 28 post-infection, respectively. For all studies (Aim 1, 2, and 3), group sizes of n = 5 for the experimental group and n = 2 for control group were used based on published JEV vaccine or challenge animal studies. In these published studies, each experimental group contained a range of 4 to 8 animals and each control group contained 2 to 3 animals at each time point to analyze their cardinal data with the appropriate statistical tests (Fan et al., 2018; Fei-fei et al., 2008; Xu et al., 2004; Yamada et al., 2004; Yamada et al., 2009; Yang et al., 2014a). Below is **Table 2.1** summarizing the experimental groups designed for Aim 1.

Table 2.1. Summary of the experimental groups for Aim 1.

Group	Intravenous inoculum	Total *n*	Necropsy at 3 DPI	Necropsy at 28 DPI
Mock	1 ml sterile PBS	n = 4	n = 2	n = 2
JEV	1 ml 10^7 $TCID_{50}$ JEV JE-91	n = 10	n = 5	n = 5

DPI = day post-infection. PBS = phosphate buffered saline. JEV = Japanese encephalitis virus.

Aim 2 research design

The study for Aim 2 was designed to investigate the impact of mosquito salivary proteins on the tropism of JEV and tissue viral loads of experimentally challenged domestic pigs, and of disease progression. A different route of infection, the intradermal route, was used to closely mimic the natural mosquito route of inoculation. A total of 28 three-week-old white-line crossbreed domestic pigs were randomly assigned into four groups (n = 4 or 10), as summarized in **Table 2.2**.

Animals were co-injected with either 100 µl equal volume mixtures of SGE and JEV stock containing 10^7 $TCID_{50}$ of JE-91 strain (SGE+JEV, *n* = 10) or 100 µl equal volume mixtures of SGE and sterile saline (SGE-only, *n* = 4). The SGE dose per pig was equivalent to 2.5 salivary gland pairs. Other groups of animals were injected with a mixture of 50 µl of sterile saline and 50 µl of JEV stock (JEV-only, *n* = 10) or 100 µl of sterile saline only (mock, *n* = 4) to characterize JEV pathological outcomes by needle inoculation. All animals were intradermally inoculated through a single injection at the base of the left ear. Prior to the injection, each pig was briefly anesthetized with isoflurane gas for about five to ten minutes to minimize distress and increase safety during the injection process. The anesthetic depth was accessed by toe pinch and jaw tone. All pigs recovered rapidly and uneventfully from the anesthesia.

The four experimental groups (mock, JEV-only, SGE-only, and SGE+JEV) were housed in separate pens. Half of the animals in each treatment group (totaling 14 animals) were sacrificed at 3 days or 28 days post-infection for the investigation of tissue tropism and viral loads during the acute and convalescent phases of JEV infection, respectively.

Table 2.2. Summary of the experimental groups for Aim 2.

Group	Intradermal inoculum	Total *n*	Necropsy at 3 DPI	Necropsy at 28 DPI
Mock	100 µl sterile PBS	*n* = 4	*n* = 2	*n* = 2
JEV-only	50 µl sterile PBS + 50 µl 10^7 $TCID_{50}$ JEV JE-91	*n* = 10	*n* = 5	*n* = 5
SGE-only	50 µl SGE + 50 µl sterile PBS	*n* = 4	*n* = 2	*n* = 2
SGE+JEV	50 µl SGE + 50 µl 10^7 $TCID_{50}$ JEV JE-91	*n* = 10	*n* = 5	*n* = 5

DPI = day post-infection. PBS = phosphate buffered saline. JEV = Japanese encephalitis virus. SGE = salivary gland extract.

Aim 3 research design

Aim 3 was conducted to establish a feral pig model for JEV. The Sinclair miniature

research swine was selected as the feral pig representative because they are an established research colony bred to have feral genetics background (Schook and Tumbleson, 1996). Originally known as the Minnesota or Hormel miniature pig, the Sinclair miniature pig was developed at the Hormel Institute of the University of Minnesota in the 1950s (Schook and Tumbleson, 1996). It is derived from breeding four feral strains (Guinea hog from Alabama, wild boar from Catalina Island, Piney Wood pig from Louisiana, and dwarf Ras-n-Lansa pig from Guam in the Mariana Islands) crossbred with the domestic Yorkshire pig (McAnulty et al., 2011). As summarized in **Table 2.3**, a total of 14 three-week-old Sinclair miniature pigs were randomly allocated into two experimental groups to be intradermally inoculated at the base of the left ear with the following: 100 µl sterile saline (mock group, $n = 4$) or 100 µl of 10^7 $TCID_{50}$ of JEV JE-91 (JEV group, $n = 10$). Similar to Aim 2 study, all pigs were briefly placed under general anesthesia using isoflurane gas for the needle injections. The two groups were housed in separate pens for the duration of study. To characterize the acute and convalescent stages of infection, groups of seven pigs (five infected and two control pigs) were sacrificed at days 3 and 28 post-infection, respectively.

Table 2.3. Summary of the experimental groups for Aim 3.

Group	Intradermal inoculum	Total *n*	Necropsy at 3 DPI	Necropsy at 28 DPI
Mock	100 µl sterile PBS	$n = 4$	$n = 2$	$n = 2$
JEV	100 µl 10^7 $TCID_{50}$ JEV JE-91	$n = 10$	$n = 5$	$n = 5$

DPI = day post-infection. PBS = phosphate buffered saline. JEV = Japanese encephalitis virus.

Sample collection and preparation

For the duration of the studies, all animals were monitored daily for any clinical signs, including fever (≥ 40 °C), depression, diarrhea, weight loss, gait abnormalities, and neurological signs. Serum and nasal swab samples were collected to characterize the kinetics of viremia and

nasal shedding, respectively. For Aim 1, whole blood samples were collected via the right external jugular vein at 0, 3, 5, 7, 14, 21, and 28 days post-infection. While more blood collection time points would have provided a better insight into the early stages of viremia (i.e. days 0 to 7 post-infection), IACUC only approved limited time points for blood collection for this study. For Aims 2 and 3, blood samples were approved to be collected daily until 7 days post-infection and then weekly until 28 days post-infection. Collected blood volumes did not exceed 1% of total blood volume of each animal due to the frequent sampling schedule. Serum samples were then obtained through the centrifugation of coagulated blood at 2,000 × *g* for 10 minutes at 4 °C and stored in -80 °C for later analysis. To characterize viral nasal shedding, individual nasal swab samples were obtained daily from 0 to 28 days post-infection from alternating nares using sterile cotton swabs and stored in 1 ml of L-15 media. They were then vortexed briefly for two to three seconds, and the swab was removed prior to centrifugation at 10,000 x *g* for 10 minutes. The supernatant was collected and stored in -80 °C for downstream analysis.

At termination days, the pigs were first sedated by intramuscular injection of 10 to 20 mg/kg of ketamine and 2 to 3 mg/kg of xylazine, and then euthanized with intravenous injection of 390 mg/ml of sodium pentobarbital via the external jugular vein. At necropsy, approximately 5 mm^3 blocks of the following tissues were collected in individual 2 mL Eppendorf tube containing 1 ml of L-15 media and a single stainless-steel homogenizing bead to characterize the viral dissemination and tissue tropism of JEV: specific regions of the brain (including olfactory bulb, olfactory peduncle, piriform cortex, midbrain, pons, medulla oblongata, cerebellum, thalamus, frontal lobe, parietal lobe, temporal lobe, occipital lobe, and caudate nucleus), spinal cord (lumbosacral region), sciatic nerve, facial nerve, olfactory neuroepithelium, nasal turbinates or epithelium, thymus, tonsil, spleen, lymph nodes (medial retropharyngeal, submandibular,

mesenteric, and/or medial iliac), Peyer's patches (small intestine), and additional tissues such as trachea, kidney, and lung. All tissue samples were stored at -80 °C prior to further processing for analyses. Prior to virus titration or RNA detection, the tubes containing the tissue samples were thawed briefly in 37 °C water bath, weighed individually, and homogenized using the TissueLyser II system (Qiagen) at 26 Hz for four minutes. They were then centrifuged at 10,000 x *g* for 10 minutes to collect the supernatant for immediate analysis.

Detection of infectious viruses

End-point dilution assay

Infectious virus titers of the stocks were determined via median tissue culture infectious dose ($TCID_{50}$) method with Vero76 cells maintained in L-15 media, as previously described (Higgs et al., 2006; Reed and Muench, 1938). The samples were first thawed in 37 °C water bath and maintained on ice. Briefly, 100 µl of each sample was loaded in duplicates in the first column of a 96-well plate and titrated in serial 10-fold dilution in L-15 media across the plate. A set of negative control (sterile L-15 media) and positive control (JEV stock with known viral titer) were included in each assay. The loaded plates were then kept on ice prior to the addition of cells. Confluent Vero76 cell culture flasks were rinsed with Mg^{2+}/Ca^{2+} free Dulbecco's phosphate buffer saline (DPBS), and the cells were removed with 0.25% trypsin-EDTA at 37 °C. The Vero76 cells were then resuspended with L-15 media with volume adjusted accordingly to the size of cell culture flask (i.e. volume of approximately 35 ml for a confluent T75 flask) and each well in the loaded 96-well plate received 100 µl of the cell culture fluid. The plates were sealed with parafilm and stored in a secondary container at 37 °C.

After seven days of incubation, the medium was removed from each plate. Each well was

fixed and stained overnight with 200 µl of cell staining dye composed of deionized water with 10% acetic acid, 25% isopropanol, and 0.1% amino black B10 stain. Lastly, the excess dye was removed and the plates were gently washed to record the titer values as $TCID_{50}$/ml based on the Reed-Muench method (Reed and Muench, 1938).

Plaque assay

To avoid the interference of detection created by the cytotoxicity of serum and homogenized tissues, plaque assay using Vero76 cells was performed to detect infectious viruses in the serum, nasal swab, and homogenized tissue samples, as previously described (Baer and Kehn-Hall, 2014; Nuckols et al., 2015). Briefly, 24-well plates were seeded with Vero76 cells using confluent cell culture flasks and left undisturbed at 37 °C for at least five hours to allow the cells to adhere to the bottom of the wells. The samples were briefly thawed in 37 °C water bath, serially diluted 10-fold with L-15 media three times to 10^{-3} dilution, and maintained on ice. Media was removed from each well of the 24-well plate containing the cells and 50 µl of the sample (undiluted, 10^{-1}, 10^{-2}, and 10^{-3} dilutions) was added per well in duplicates. A set of negative control (sterile L-15 media) and positive control (JEV stock with known viral titer) were included in each assay. The inocula adsorbed at 37 °C for 45 minutes with gentle agitation of the plate every 10 to 15 minutes. After the adsorption period, the inoculum was removed and the wells were rinsed with 500 µl of DPBS/well prior to adding 1 ml of 1.5% methyl cellulose overlay per well. The plates were sealed with parafilm and left to incubate at 37 °C.

The 1.5% methyl cellulose overlay was prepared by autoclaving a 500 ml round media bottle in liquid setting containing the following: 7 to 8 g of methyl cellulose, 280 ml of molecular biology grade sterile water, and a magnetic stir bar. After completion, 50 ml of chilled TPB, 250

ml of chilled 2x L-15 media, 30 ml of FBS, and 10 ml of antibiotics and L-glutamine mixture (100 U/ml penicillin, 100 µg/ml of streptomycin, and 2 mM of L-glutamine) were added to the bottle. Using a magnetic stirrer, it was left to spin for 10 hours or more until the media became uniformly viscous. The overlay was then stored in 4 °C and tested for sterility at 37 °C prior to use.

After five days of incubating the loaded 24-well plates, each well was fixed with 1 ml of 10% formalin solution (formaldehyde diluted in PBS) for 30 minutes. The overlay and fixative mixture were removed, and each well was stained for at least five minutes with 1% crystal violet solution (composed of deionized water with 0.5% crystal violet powder and 50% methanol). After gently washing the plates, the plaques were counted and the titers of infectious viruses were calculated in plaque forming units (PFU)/ml or PFU/g, as shown in **Figure 2.1**.

$$\frac{(Number\ of\ plaques)}{(10^{dilution})(0.050\ ml)} = \frac{PFU}{ml}$$

$$\frac{(Number\ of\ plaques)}{(10^{dilution})(0.050\ ml)(\frac{tissue\ weight\ in\ g}{1\ ml})} = \frac{PFU}{g}$$

$$Dilution = 0, -1, -2, or -3$$

Figure 2.1. Formulas used to calculate the viral titer in PFU/ml or PFU/g.
PFU = plaque forming units.

Detection of viral genome

The presence of JEV was further confirmed and detected by a more sensitive method of RT-qPCR. It was also important to verify the identity of the viral plaques because the animals were not specific-pathogen-free of other virus infections. Genome equivalents of JEV in serum, nasal

swabs suspensions, and homogenized tissues were determined using a previously published TaqMan one-step RT-qPCR assay targeting the genomic fragment encoding the nonstructural protein 5 (Pyke et al., 2004). Viral RNA was first extracted from the serum and nasal swab suspension using the QIAamp viral RNA extraction kit (Qiagen). Total RNA was extracted from the homogenized tissue samples with Trizol LS (Invitrogen) following the manufacturer's instructions.

For each sample reaction, the iTaq Universal Probe One-step kit (Bio-Rad) was used to prepare 20 µl total reaction mixture containing the following: 10 µl of 2x iTaq Universal Probes one-step reaction mix, 0.5 µl of iScript Reverse Transcriptase, 10 pmol (1 µl of 10 µM) of forward primer (5′ATCTGGTGYGGYAGTCTCA3′), 10 pmol (1 µl of 10 µM) of reverse primer (5′CGCGTAGATGTTCTCAGCCC3′), 4 pmol (0.4 µl of 10 µM) of 5′ 6-carboxyfluorescein (FAM)-labeled probe with internal ZEN and 3′ tetramethylrhodamine quencher (5′FAM-GGAACGCGATCCAGGGCAA-IABkFQ3′), 3.1 µl of molecular grade water, and 4 µl of RNA sample. Reactions were performed on the CFX96 Real-Time PCR Detection System (Bio-Rad) with the cycling parameters described by Pyke et al. (2004), as shown in **Table 2.4**.

Table 2.4. Cycling parameter for the selected RT-qPCR assay, as established by Pyke et al. (2004).

Step	Temperature and time setting
1	50 °C for 30 minutes
2	95 °C for 3 minutes
3	95 °C for 15 seconds
4	48 °C for 3 minutes
5	Repeat cycling 44 times between Step 3 and Step 4

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 8.52 $\log_{10}TCID_{50}$/ml (**Figure 2.2**). A negative blank

control was included for each reaction. Results were reported as genome equivalent to $log_{10}TCID_{50}$/ml (geq-$TCID_{50}$/ml) or $log_{10}TCID_{50}$/g (geq-$TCID_{50}$/g). Samples were considered positive when the Ct value was lower than 34.

	A	B	C	D	E	F	H	I	J	K	L
1		Well	Fluor	Target	Content	Sample	Cq	Cq Mean	Cq Std. Dev	Starting Quantity (SQ)	Log Starting Quantity
2		A01	FAM		Std		7.59	7.59	0.000	100000000.00000	8.000
3		A02	FAM		Std		8.27	8.27	0.000	100000000.00000	8.000
4		B01	FAM		Std		11.40	11.40	0.000	10000000.00000	7.000
5		B02	FAM		Std		11.32	11.32	0.000	10000000.00000	7.000
6		C01	FAM		Std		14.73	14.73	0.000	1000000.00000	6.000
7		C02	FAM		Std		15.26	15.26	0.000	1000000.00000	6.000
8		D01	FAM		Std		18.59	18.59	0.000	100000.00000	5.000
9		D02	FAM		Std		20.06	20.06	0.000	100000.00000	5.000
10		E01	FAM		Std		22.01	22.01	0.000	10000.00000	4.000
11		E02	FAM		Std		23.74	23.74	0.000	10000.00000	4.000
12		F01	FAM		Std		25.89	25.89	0.000	1000.00000	3.000
13		F02	FAM		Std		27.43	27.43	0.000	1000.00000	3.000
14		G01	FAM		Std		29.14	29.14	0.000	100.00000	2.000
15		G02	FAM		Std		30.92	30.92	0.000	100.00000	2.000
16		H01	FAM		Std		38.31	38.31	0.000	10.00000	1.000
17		H02	FAM		Std			0.00	0.000	10.00000	1.000

Figure 2.2. Sample output of standard curve generated per each reaction.

Plaque reduction neutralization test

To determine the neutralizing antibody titers, plaque reduction neutralizing tests (PRNT) were performed following the procedures described by Roehrig et al. (2008). All serum samples were first heat inactivated at 56 °C for 30 minutes and then serially diluted 2-fold starting at 1:10 to 1:640 dilutions in deep 96-well plates. Approximately 75 PFU of JEV JE-91 strain was added to each serum concentration and incubated for 1 hour at 37 °C prior to infection of Vero76 cells in six-well plates. Media was removed from the 6-well plates containing the cells and 200 µl of each serum-virus mixture was added per well in duplicates. A set of negative control (sterile L-15 media) and virus only control was included in each assay. The adsorption process and the rest of the procedure for PRNT followed the same protocol described for the plaque assay. After an

adsorption period of 45 minutes at 37 °C, the wells were gently washed with DPBS and overlaid with 1% methyl cellulose. The plates were then sealed with parafilm and stored at 37 °C.

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

Statistical analyses

The SAS software (version 9.4, SAS Institute, Cary, NC) and SPSS Statistics software (IBM) were used for all statistical analyses. The R software (versions 3.4.1 to 4.1.0, The R Foundation) was used for data graphical display. All statistical analyses were performed on raw data. Shapiro-Wilk test was used to test the normality of raw and log-transformed data. Viral loads in tissue samples collected from the experimental groups were evaluated by nonparametric Kruskal-Wallis tests with post hoc Dunn's multiple pairwise comparison test adjusted with Bonferroni correction (Dunn-Bonferroni test) and post hoc Mann-Whitney U tests. Owning to the violation of normality assumption and considering time as a factor, nonparametric Kruskal-Wallis tests and post hoc Dunn-Bonferroni tests were performed to compare temperature, viremia levels, and nasal shedding levels between the groups when appropriate. Mann-Whitney U tests were used to compare antibody titers and onset of ataxia between two virus-challenged groups when applicable. For the differences in the duration of nasal shedding between virus-challenged groups, Student's *t*-test was used for such an evaluation. Fisher's exact tests were used to analyze the difference in fever, nasal shedding, and ataxia incidence between the virus-challenged groups when appropriate.

Chapter 3 - Susceptibility of North American domestic pig to JEV infection via intravenous challenge

The objective of Aim 1 was to determine the susceptibility of North American domestic pigs to JEV through the invasive challenge of intravenous inoculation to test the working hypothesis that North American domestic pigs are susceptible to JEV infection. The intravenous route was selected because it is an efficient delivery method of introducing the pathogen into the animal to induce viremia and observe if pathology or disease can be established. The work displayed here in this chapter has been published by Springer Nature in *Scientific Reports* journal, available online at https://doi.org/10.1038/s41598-018-26208-8. All manuscript sections have been altered from when it was originally published and has not undergone peer-review.

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Introduction

Previous studies have identified competent mosquitoes and susceptible avian species in North America that can sustain the enzootic transmission of JEV, designating the pathogen as a

significant health threat (Huang et al., 2016a; Huang et al., 2015; Nemeth et al., 2012). Despite the significant role that pigs play in JEV-endemic regions as efficient amplifying hosts (Platt and Joo, 2006; van den Hurk et al., 2009), the susceptibility of North American swine to JEV infection and its disease outcomes remains largely unknown. At the time when these dissertation studies were conducted starting in 2016, several pathogenesis studies have been performed with pigs derived from endemic regions, as summarized in **Table 3.1**. However, the results from these JEV challenge experiments cannot be extrapolated to directly demonstrate the degree of susceptibility to JEV among the domestic pigs in North America used for swine and pork production. This is mainly because disease resistance or susceptibility can vary based on the breed or population locality of the animals, which has been demonstrated in pigs for several viral infections (Blacksell et al., 2006; Meng et al., 2018; Mujibi et al., 2018; Opriessnig et al., 2006). Additionally, the majority of the published studies were performed with JEV strain belonging to genotype III, which was previously dominant in the endemic region but has been displaced by the rapidly emerging strains under the clade b of genotype I (Desingu et al., 2016; Ilkal et al., 1994; Pan et al., 2011; Williams et al., 2001; Yamada et al., 2004). Such a gap of knowledge precludes the comprehensive assessment needed to estimate the risk and develop effective countermeasures against the potential emergence of JEV in the United States.

Table 3.1. Examples of previous JEV challenge studies of pigs.

Animals	Challenge routes and inocula	Major finding(s)	Reference
Pregnant (gestation day 36 to 97) Yorkshire hybrid pigs (groups of *n* = 1)	IV with 10^6 to 10^9 mouse LD_{50} of JEV Fuji or Kanagawa strains (GIII)	• JEV is a causative agent of reproductive failure (stillbirths) in infected pregnant swine	(Shimizu et al., 1954)
10- to 20-day-old piglets of local breed from India (groups of *n* = 3-4)	SQ with 10^4 to 10^5 mouse LD_{50} JEV 897795 strain; bite by JEV-infected *Culex vishnui* (genotype undetermined)	• WNV infection provided partial cross-reactive immunity in pigs against JEV	(Ilkal et al., 1994)
6-week-old domestic outbred pigs (groups of *n* = 3)	SQ with 10^5 to 10^6 $TCID_{50}$ JEV Nakayama strain (GIII)	• Cross-reactive immunity against MVEV and KUNV was demonstrated in pigs after JEV infection	(Williams et al., 2001)
3-week-old SPF piglets (groups of *n* = 1-3)	IV with ~10^6 $TCID_{50}$ JEV IB 2001 or AS-6 strains (genotype undetermined)	• JEV-induced encephalitis in pigs was characterized • Immunohistochemical distribution of viral antigens of JEV and the neurotropism of JEV were demonstrated in JEV-infected pigs	(Yamada et al., 2004)
7-week-old Swiss Large white pigs (groups of *n* = 2-3)	ID/IV with 10^6 to 10^7 $TCID_{50}$ JEV Nakayama strain (GIII); Oronasal with 10^3 to 10^7 $TCID_{50}$ Nakayama; ID or IV with 10^6 $TCID_{50}$ JEV Laos strain (GI)	• Vector-free transmission of JEV was demonstrated experimentally in pigs • Similar pathogenesis can be observed regardless of the different modes of infection and JEV genotype	(Ricklin et al., 2016a)
7-week-old Swiss Large white pigs (groups of *n* = 3)	ID/IV with ~10^7 $TCID_{50}$ Nakayama strain (GIII)	• The tissue dissemination pattern of JEV in pigs was determined • JEV has tropism for both CNS and lymphoid tissues in pigs	(Ricklin et al., 2016b)

IV = intravenous. ID = intradermal. SQ = subcutaneous. JEV = Japanese encephalitis virus. MVEV = Murray Valley encephalitis virus. KUNV = Kunjin virus. G = genotype. DPI = days post-infection. SPF = specific pathogen free. CNS = central nervous system.

The first step in addressing this research gap was to conduct a challenge study using the intravenous challenge. In this study, the common North American white-line crossbreed of domestic pigs were intravenously inoculated with a representative strain for genotype Ib to determine their susceptibility to the newly emerging genotype of JEV. Fourteen three-week-old piglets were inoculated with JE-91 (JEV genotype Ib strain) at approximately 10^7 $TCID_{50}$ (n = 10) or sterile saline (n = 4) and euthanized at day 3 or 28 post-infection to characterize the acute and convalescent phases of infection, as illustrated in **Figure 3.1.** The intravenous challenge route and dose were selected because of the pathological findings described in other experiments utilized the similar approach, as detailed in **Table 3.1**. This will allow the comparison of susceptibility and infection outcomes between domestic pigs in North America and other regions. Most importantly, the intravenous route was chosen as it is an efficient and invasive route of injection to introduce the pathogen to the animal and observe if pathology, disease, and/or clinical signs will develop. The direct delivery of the infectious viruses into blood will induce viremia needed to support the hematogenous route of viral dissemination and help determine if infection and disease can be established. Data from this study will serve as preliminary findings to support the future experiments using routes of experimental challenge that resemble natural infections.

Pathogenic outcomes and tissue tropism were characterized by detection of infectious viruses and viral genomes. Challenged animals developed detectable levels of viremia, systemic spread through lymphoid tissues, oronasal shedding, neuroinvasion, and viral persistence in the tonsils, suggesting that North American pigs are susceptible to JEV and are capable of sustaining its enzootic transmission cycle in the event of its introduction.

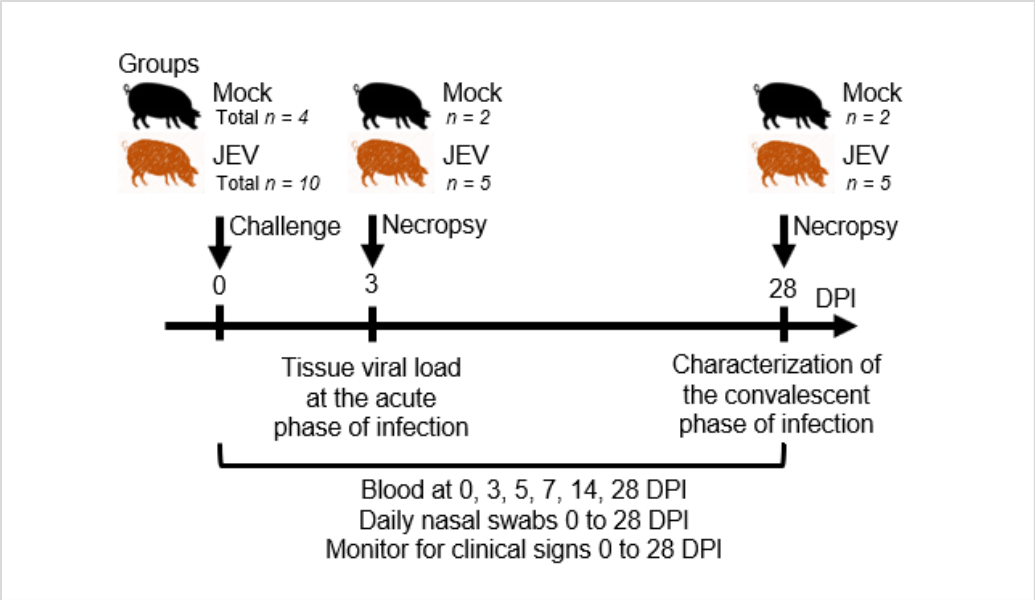

Figure 3.1. Schematic diagram of Aim 1 experimental design.
DPI = day post-infection. JEV = Japanese encephalitis virus.

Results

Clinical outcomes and viremic profiles of JEV-infected pigs

The animals were healthy and seronegative to JEV prior to the experimental challenge. Fever, weight loss, depression, lethargy, and hind limb ataxia were observed in JEV-infected pigs after inoculation, but most clinical signs resolved within one week. All JEV challenged pigs (n = 10) demonstrated nonspecific clinical signs, with three exhibiting mild hind limb ataxia and gait abnormalities. High fevers up to 41 °C were observed in infected pigs as early as day 1 post-infection and lasted four to five days before temperatures decreased to within normal limits (<40 °C). Although not statistically significant, minor weight loss was recorded in 50% (5/10; Mann-Whitney U test, p = 0.454) of infected pigs between 1 and 2 days post-infection and in all infected pigs (10/10; Mann-Whitney U test, p = 0.635) between 3 and 4 days post-infection. Challenged pigs also exhibited depression and lethargy since day 1 after challenge. While all

returned to bright, alert, and responsive states after day 5 post-infection, one pig continued to be slightly depressed until 13 days post-infection. The same animal also developed a second fever peak of 40.4 °C on day 8 post-infection that resolved in three days. Mild ataxia in the rear legs was then subsequently observed between 10 to 13 days post-infection. Two other infected pigs also exhibited gait abnormalities of the rear legs on day 19 post-infection that resolved by day 27 post-infection.

Viremia was detected in all of the infected animals tested. **Figure 3.2** summarizes the viral titers of serum samples collected at day 3 post challenge. Serum samples from two infected pigs showed detectable levels of infectious viruses by plaque assay, reaching up to $2.0x10^1$ PFU/ml (**Figure 3.2a**). The presence of JEV was further confirmed and detected by RT-qPCR. It was also important to verify the identity of the viral plaques because the pigs in this study were not specific-pathogen-free of other virus infections. Results from RT-qPCR demonstrated that at least eight animals developed viremia at day 3 post-infection with viral RNA loads ranging between $1.34x10^2$ and $4.2x10^3$ geq-$TCID_{50}$/ml (**Figure 3.2b**). Serum viral load of 6.07 geq-$TCID_{50}$/ml was detected in one challenged animal at day 5 post-infection. These results suggest that clearance of viremia in domestic pigs can take place as early as three days after intravenous challenge. The recovery from the acute phase of infection was also demonstrated as all animals developed neutralizing antibodies at a geometric mean titer of 1:243 at 28 days after challenge.

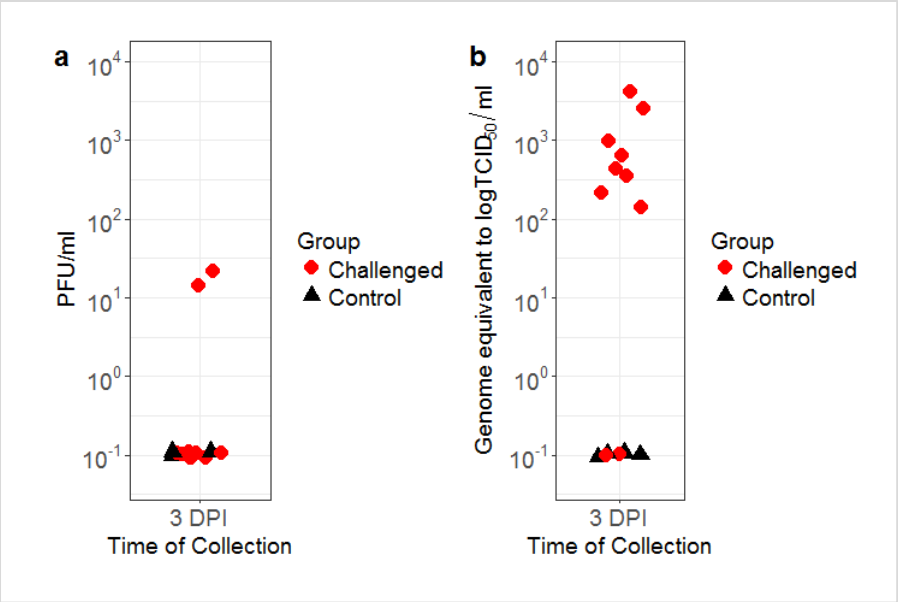

Figure 3.2. Viremia at day 3 post-infection.

Viral titers of serum collected at day 3 following JEV challenge quantified by plaque assay (a) and RT-qPCR (b). PFU = plaque forming units. DPI = day post-infection.

Viral shedding in nasal secretions

To characterize the nasal shedding dynamics of JEV in pigs, secretions from the nose were collected daily from alternating nares for virus titration. Infectious virus was detected in the nasal swab samples by day 2 post-infection for up to five days, as shown in **Figure 3.3a**. Up to 90% (9/10) of the infected pigs were actively shedding infectious virus at various time points for a period of one to five days. At 3 days post-infection, the highest infectious titer was observed at $4.8x10^2$ PFU/ml. About 60% (3/5) of infected pigs continued to shed viruses to day 4 post-infection. Shedding of infectious virus persisted for up to six days in an infected pig. By 7 days post-infection, no nasal swabs were positive for JEV.

Similar shedding kinetics were observed via RT-qPCR, as summarized in **Figure 3.3b**. JEV shedding was detectable at day 2 post-infection, at average titers of $1.6x10^1$ geq-$TCID_{50}$/ml. At day 3 post-infection, 80% (8/10) of the challenged pigs shed between 5.62 and $8.18x10^2$ geq-

$TCID_{50}$/ml. A peak titer of $4.76x10^4$ geq-$TCID_{50}$/ml was detected at 4 days post-infection. While most challenged animals stopped shedding after day 6 post-infection, viral shedding of up to $1.4x10^1$ geq-$TCID_{50}$/ml was detectable from one animal up to 10 days post-infection.

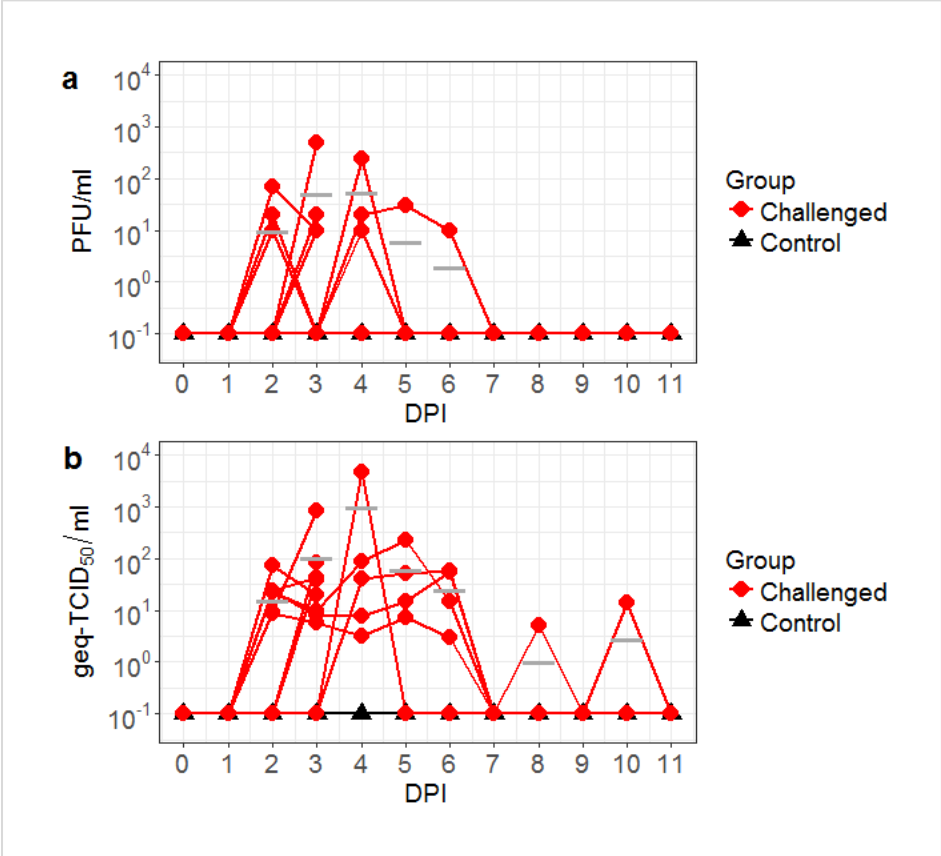

Figure 3.3. Kinetics of viral nasal shedding after intravenous JEV challenge.

Nasal shedding of JEV by experimentally infected pigs quantified by plaque assay (a) and RT-qPCR (b). PFU = plaque forming units. DPI = day post-infection. Geq-$TCID_{50}$ = genome equivalent-50% tissue culture infectious dose. Bar lines indicate the mean of the values collected from the challenged animals.

Dissemination of JEV at the acute phase of infection

The dissemination of JEV at the acute phase of infection were determined by the titration of tissue samples harvested at day 3 post-infection. The presence of infectious virus in the central

nervous system (CNS) and peripheral nervous tissues demonstrated that infection of JEV can lead to neuroinvasion among North American domestic pigs. As shown in **Figure 3.4a**, infectious virus was recovered from six nervous tissue samples (facial nerve, olfactory bulb, olfactory neuroepithlium, optic nerve, piriform cortex, and thalamus) with titers ranging from $5.0x10^1$ PFU/g to $1.9x10^2$ PFU/g. Infectious virus was present in the olfactory neuroepithelium of 60% (3/5) of infected pigs, reaching titers up to $2.1x10^3$ PFU/g but not statistically significantly higher (Dunn-Bonferroni test, $p \geq 0.999$) compared to other positive neural tissues.

As summarized in **Figure 3.4b**, infectious virus was also detected in the lymphatic system of challenged animals, indicating the systemic spread of JEV at the acute phase of infection. Mesenteric lymph nodes and spleen of all challenged animals were positive for infectious viruses at average titers of $3.1x10^3$ PFU/g and $9.0x10^2$ PFU/g, respectively. Mesenteric lymph nodes had significantly higher viral titers (Dunn-Bonferroni test, $p = 0.005$) than most other positive neural tissues. The presence of infectious virus was also observed in the tonsil of one animal at the titer of $7.3x10^3$ PFU/g. Dissemination of JEV was observed in other tissues including the trachea, lungs, and kidneys (**Figure 3.4c**). Out of the positive peripheral tissues, nasal epithelium had a particularly high mean infectious viral titer of $7.53x10^2$ PFU/g, with a peak of $2.6x10^3$ PFU/g in one challenged pig but the infectious titers of the nasal epithelium were not statistically significant (Dunn-Bonferroni test, $p \geq 0.999$). Other CNS, lymphoid, and visceral tissues including different regions of the cerebral cortex, brainstem, cerebellum, spinal cord, Peyer's patches, liver, skeletal muscle, and reproductive tract, did not contain detectable amounts of infectious viruses.

The systemic infection and neuroinvasion of JEV were demonstrated by JEV-specific RT-qPCR, as depicted in **Figure 3.5**. Analysis by RT-qPCR was again needed to validate the results from the plaque assay. As expected, the RT-qPCR assay used in this study provided a higher

sensitivity than plaque assay in detecting the presence of JEV. Consistent with the results of plaque assays, homogenized olfactory neuroepithelium had the highest viral load at $1.8x10^6$ geq-$TCID_{50}$/g and overall had higher viral loads than most neural tissues (**Figure 3.5a**). However, the viral loads detected from the olfactory neuroepithelium were not statistically different from those of other tissues (Dunn-Bonferroni test, $0.502 \leq p \leq 0.999$). The lowest mean titer of $4.4x10^1$ geq-$TCID_{50}$/g was recovered from the sciatic nerve. Other notable CNS structures with average viral loads above 10^3 geq-$TCID_{50}$/g included the cerebellum ($1.6x10^3$ geq-$TCID_{50}$/g), thalamus ($1.1x10^3$ geq-$TCID_{50}$/g), temporal lobe ($1.1x10^3$ geq-$TCID_{50}$/g), and frontal lobe ($1.0x10^3$ geq-$TCID_{50}$/g). However, these structures were not statistically different from one another (Dunn-Bonferroni test, $p \geq 0.999$).

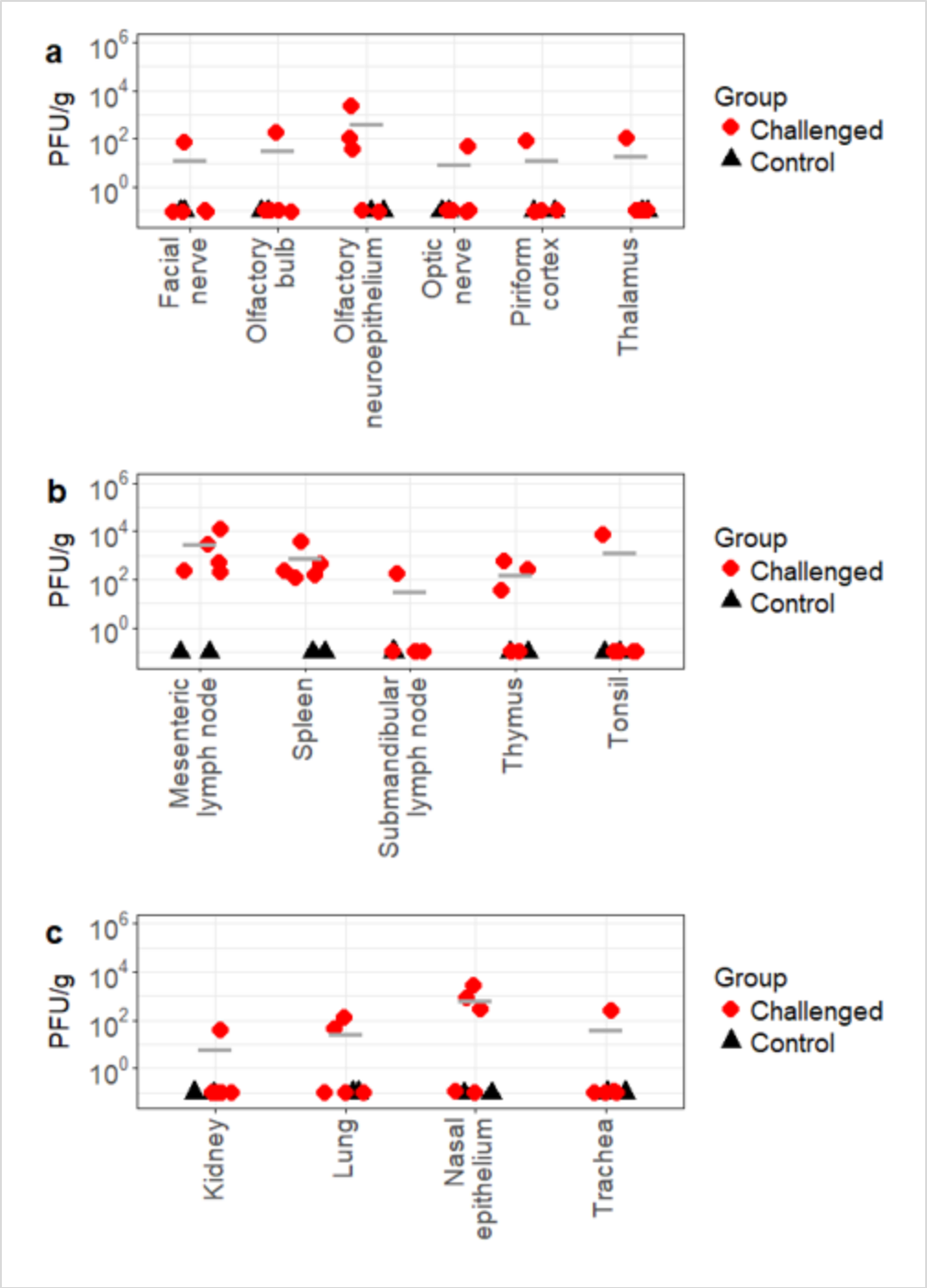

Figure 3.4. Tissue dissemination pattern of JEV determined by plaque assay.

Infectious viral titers of JEV-positive CNS (a), lymphoid (b), and other (c) tissues collected at day 3 post-infection. PFU = plaque forming units. Bar lines indicate the mean of the values collected from the challenged animals.

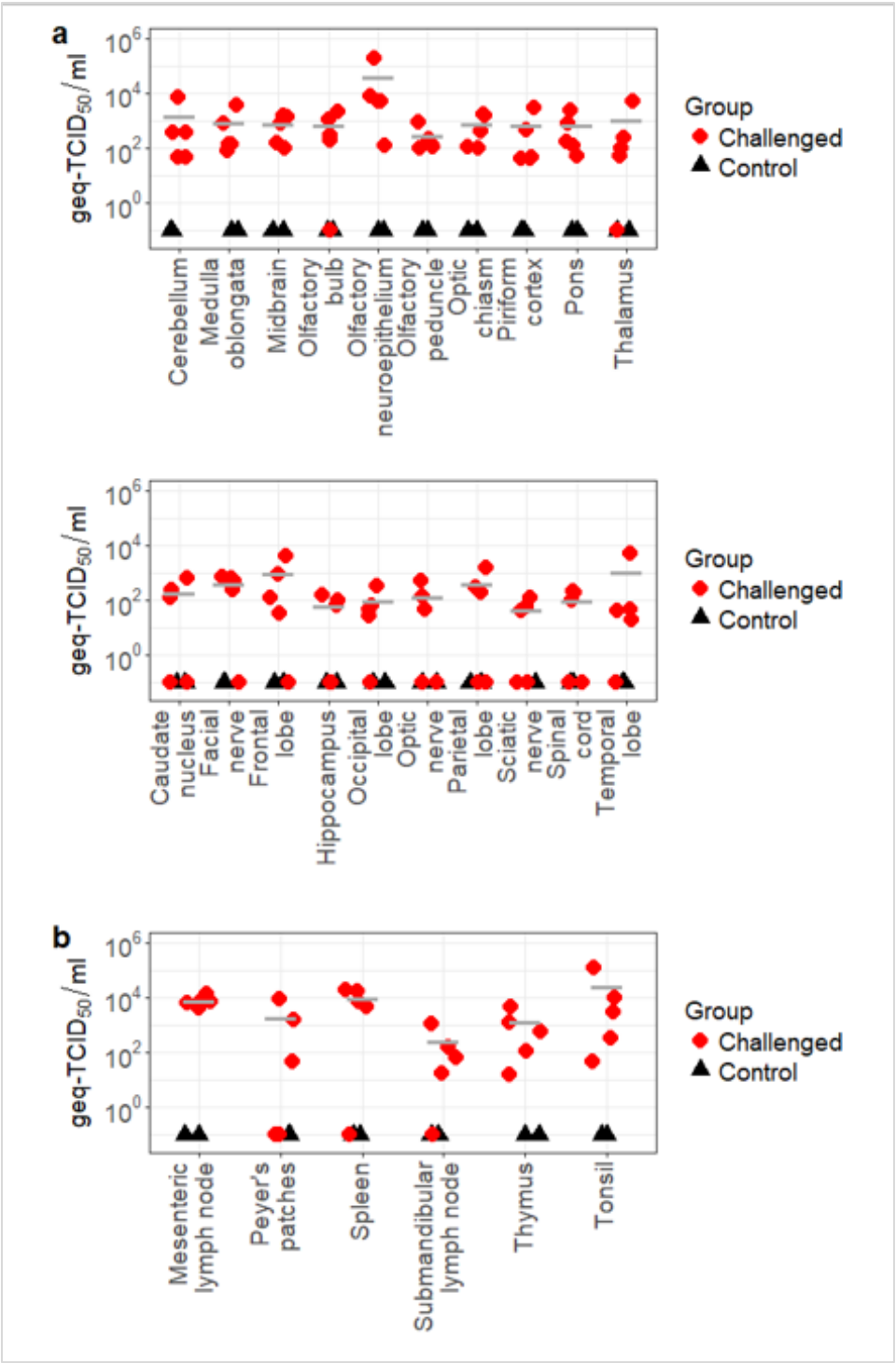

Figure 3.5. Tissue dissemination pattern of JEV determined by RT-qPCR.

Viral load of CNS (a) and lymphoid (b) tissues collected at day 3 post-infection, as estimated by RT-qPCR. Geq-$TCID_{50}$ = genome equivalent-50% tissue culture infectious dose. Bar lines indicate the mean of the values collected from the challenged animals.

Among the lymphoid structures of the infected pigs, the submandibular lymph nodes had the lowest viral load ranging from $1.7x10^1$ to $1.0x10^3$ geq-$TCID_{50}$/g (**Figure 3.5b**). Similar to the infectious viral titer results, tonsils, mesenteric lymph nodes, and the spleen produced the highest average viral RNA titers of $2.6x10^4$ geq-$TCID_{50}$/g, $7.6x10^3$ geq-$TCID_{50}$/g, and $9.7x10^3$ geq-$TCID_{50}$/g, respectively. However, there was no demonstrable statistical difference in viral RNA loads between the lymphoid tissues (Dunn-Bonferroni test, $p \geq 0.999$). The same infected pig that produced the highest viral load in the olfactory neuroepithlieum also produced the highest viral load in the tonsils, which reached $1.1x10^5$ geq-$TCID_{50}$/g. In summary, multiple nervous and lymphatic tissues showed the positive detection of viral RNA by RT-qPCR. These results demonstrate the high incidence of neuroinvasion and systemic infection among the animals challenged with JEV.

Viral clearance and persistent infection of JEV

Titration of homogenized tissues collected from the five challenged animals at 28 days post-infection failed to detect any infectious viruses. However, viral genome was detected by RT-qPCR in the tonsils. Viral loads of tonsils collected from three infected pigs ranged between $4.9x10^1$ to $3.4x10^2$ geq-$TCID_{50}$/g, indicating that there was active ongoing viral replication occurring in this structure although no live viruses could be isolated by plaque assay. It could also suggest the possibility of the presence of RNA fragments with no active replication.

Discussion

The results from this study demonstrate that North American domestic pigs, as used for commercial pork production, are susceptible to JEV infection. North American pigs infected with

genotype Ib JEV JE-91 strain developed nonspecific clinical signs including depression, fever, and minor weight loss. The disease course was then followed by mild to moderate bilateral hind limb ataxia, which is a clinical finding often reported with other ambulatory abnormalities in horses infected with JEV (Gulati et al., 2011; Sellon and Long, 2007). Previous experimental challenge studies with pigs from Asia and Europe inoculated with genotype III virus strains reported similar clinical signs (Ricklin et al., 2016b; Shimizu et al., 1954; Yamada et al., 2004; Yamada et al., 2009), although neurologic signs such as hind limb tremors were only documented in the studies conducted in Japan (Yamada et al., 2004; Yamada et al., 2009). This suggests that JEV susceptibility between pigs from different regions and/or the infection course of genotypes III and Ib in pigs may not be significantly different from each other. Nonetheless, with North American pigs being susceptible to JEV, an enzootic JEV transmission cycle can easily become established in North America because there is no pre-existing immunity in their pig population to the foreign virus.

Clinical disease was also coupled with viremia and viral shedding. Viral titers as high as $4.2x10^3$ geq-$TCID_{50}$/ml were detected at day 3 post-infection in the serum of infected North American pigs (**Figure 3.2b**). This amount of virus in the blood is slightly lower compared to previous reports, in which higher than 10^4 infectious virus quantities per ml were reported (Gresser et al., 1958; Konishi et al., 1992; Ricklin et al., 2016a; Ricklin et al., 2016b). As such, while viremia was demonstrated in the infected pigs, whether the peak was caught at day 3 post-infection following intravenous challenge is unclear. Nonetheless, our reported viremias based on genome equivalent data may be sufficient to infect feeding mosquitoes (Platt and Joo, 2006; Takahashi, 1976). For example, albeit the low infection rates, the highly JEV susceptible *Cx. tritaeniorhynchus* mosquitoes can become infected *per os* at infectious titers as low as $10^{1.5}$ LD_{50}

(Raengsakulrach et al., 1999; Sasaki et al., 1982; Takahashi, 1976). The event of subsequent transmissions to other susceptible vertebrate hosts is possible in the presence of highly susceptible mosquito species. The minimal infective dose for JEV competent mosquitoes found in North America, such as *Cx. quinquefasciatus*, remains to be determined. In the meantime, transmission in the absence of competent vectors may potentially also occur between susceptible vertebrates based on recent findings on the significance of nasal shedding of JEV (Ricklin et al., 2016a). While most infected pigs in this study shed an average viral titer of $7.2x10^1$ PFU/ml or $2.25x10^2$ geq-$TCID_{50}$/ml in the nasal secretions (**Figure 3.3**), Ricklin et al. (2016a) demonstrated that viral titers as low as 10 $TCID_{50}$/ml can be infectious to pigs via the intranasal route. Therefore, once a pig becomes infected, animal-to-animal transmission may occur throughout the entire herd. The risk for vector-free aerosol or contact transmission of JEV from pigs to humans is currently unknown, but intranasal infection of JEV has been demonstrated in other vertebrate species including rhesus monkeys (Raengsakulrach et al., 1999), macaques (Myint et al., 2014; Myint et al., 1999), and mice (Li et al., 2012; Tsuchiya, 1968).

In terms of tissue tropism and virus dissemination, JEV behaved similarly as reported in previous published studies (Bosco-Lauth et al., 2011; Li et al., 2017a; Ricklin et al., 2016b; Yuan et al., 2016) and displayed tropism for nervous and lymphoid tissues in North American pigs. Titration and quantification of viral RNA via RT-qPCR of homogenized tissue samples identified the following tissues with the highest viral titers at 3 days post-infection: nasal epithelium, olfactory neuroepithelium, mesenteric lymph node, spleen, and tonsil. The highest values from these structures ranged from $2.1x10^3$ PFU/g to $1.2x10^4$ PFU/g or $3.5x10^3$ geq-$TCID_{50}$/g to $3.6x10^4$ geq-$TCID_{50}$/g (**Figures 3.4** and **3.5**). Such high titers at the acute stage of infection, particularly in the nasal epithelium and olfactory epithelium, highlight two significant findings. Firstly, the source

of nasal shedding may be attributed to virus replicating in either the nasal epithelium or olfactory neuroepithelium, although JEV antigens could not be detected in these structures in Asian pigs after JEV intranasal challenge in a previous study (Yamada et al., 2009). Secondly, the high viral titers detected in the olfactory neuroepithelium at the acute stage of infection provides support to the previous finding that JEV can reach the brain through the olfactory pathway in pigs (Yamada et al., 2009). Similarly to alphaviruses capable of causing encephalitis such as Sindbis (Cook and Griffin, 2003) and Venezuelan equine encephalitis (Ryzhikov et al., 1995) and encephalitic flaviviruses such as St. Louis encephalitis (Monath et al., 1983) and Murray Valley encephalitis viruses (McMinn et al., 1996), JEV can bypass the blood-brain-barrier to reach the brain by retrograde axonal transport through the olfactory neuroepithelium in addition to the hematogenous route of brain infection described in other studies (Clark et al., 2012; Yamada et al., 2004). Since JEV neuroinvasion is regarded to be age-dependent (Clark et al., 2012; Grossberg and Scherer, 1966; Kimura et al., 2013), it would be interesting to investigate if this pattern of viral infection and dissemination is also observed in adult pigs, which reportedly only experience reproductive disease from JEV infection (Mansfield et al., 2017; Platt and Joo, 2006).

Another significant finding in this study was viral persistence in the tonsils. While no infectious virions could be isolated, viral RNA loads approximately 10^1 to 10^2 geq-$TCID_{50}$/g were detected at day 28 post-infection in the tonsils of infected North American pigs. This discrepancy between the plaque assay and RT-qPCR may have occurred because the amount of live infectious viruses in the tonsil were below the limit of detection of cell-based detection methods like plaque assay. Nonetheless, comparable results were also observed in an European study, in which upwards of 10^4 RNA units equivalent to $TCID_{50}$/g of JEV were detected at 25 day post-infection in the tonsils of their local domestic pigs after needle-challenge (Ricklin et al., 2016a). In a more

recent study, viral RNA was detected in the tonsil for at least 46 days after challenge, suggesting that the virus may be somehow hidden or evading from the host immune system (García-Nicolás et al., 2017). This is an important finding, because this may indicate that pigs could remain as potential carriers for at least a month after the initial infection, further emphasizing the significant role that pigs play in JEV transmission. Whether or not this persistent infection can lead to the reactivation of viremia or nasal shedding later on remains undetermined. However, other animal viruses that persistently infect tonsils, such as bovine herpesvirus 1 (Winkler et al., 2000) and porcine reproductive and respiratory syndrome virus (Bierk et al., 2001; Pileri and Mateu, 2016), have been documented to be able to reactivate and cause secondary infections. With JEV, reactivation of latent and persistent infection has been documented in mouse models (Mathur et al., 1986a, b) and in human cases (Sharma et al., 1991) based on the isolation of infectious virus weeks after the initial infection. Therefore, persistent infection of JEV in pigs warrants further investigation as it can have potential significant implications to disease transmission and control. Pigs can potentially become a model for persistent flavivirus infections.

It is also important to interpret the results of this study with caution. First, the present study demonstrated that juvenile pigs in North America are susceptible to JEV. While young piglets of the common domestic white-line crossbreed were used as representative pigs of North America, the observations may not be directly extrapolated to the disease pathogenesis of JEV in adult pigs. However, their susceptibility to JEV remains important and relevant as there are continuously stable populations of young piglets available due to the high turnover rate of pigs in swine and pork production. Second, although intravenous injection does not mimic the natural route of transmission, it allows the comparison of susceptibility and infection outcomes between domestic pigs in North America and other regions as other challenge experiments have used similar

approaches (Ricklin et al., 2016a; Ricklin et al., 2016b; Shimizu et al., 1954; Yamada et al., 2004). Moreover, Ricklin et al. (2016a) reported that the different modes of infection used in their study (i.e. intradermal/intravenous combination and intranasal) did not result in fundamental differences in CNS lesions or tropism and level of neutralizing antibody titers.

Collectively, this study demonstrates for the first time that North American domestic pigs can contribute to the JEV transmission cycle as amplifying hosts. Along with the evaluations of North American mosquitoes (Huang et al., 2016a; Huang et al., 2015; Reeves and Hammon, 1946) and avian species to JEV infection (Nemeth et al., 2012), the present study further highlights that there are competent mosquito vectors and susceptible amplifying hosts present in North America that can support and maintain JEV transmission. As such, JEV may have the potential to become endemic in the United States after an introductory event similar to the recent emergence of West Nile virus, a closely related flavivirus (Ciota and Kramer, 2013). With this potential risk, it is important to continue the international surveillance of JEV and possibly also locally in the United States by implementing JEV diagnostic methods, such as antibody or viral RNA detection, into the standard work up for quick identification and response as JEV is both a significant swine and human pathogen that cannot be ignored.

Chapter 4 - Effect of mosquito saliva in JEV pathogenesis in the domestic pig

The intravenous challenge study in Aim 1 demonstrated the susceptibility of North American pigs to JEV, but the inoculation method did not resemble the natural route of infection. The next step in characterizing JEV infection and disease in North American pigs was to mimic the natural route of JEV transmission more closely via intradermal inoculation in Aim 2. Using the intradermal route of challenge will provide a better view into the pathogenesis of JEV. This will also allow us to assess the roles of mosquito saliva in JEV infection. The main objective of this study was to investigate the effect of mosquito saliva in JEV pathogenesis of North American domestic pigs to test the working hypothesis that the addition of mosquito saliva in the needle inoculation of JEV will modulate the virus replication and/or disease. The work displayed here in this chapter has been published in the *Emerging and Reemerging Viruses* section of the *Frontiers in Virology* journal. It is available online at https://doi.org/10.3389/fviro.2021.724016. All manuscript sections have been altered from when it was originally submitted and has not undergone peer-review.

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Introduction

Japanese encephalitis virus is transmitted by *Culex* species mosquitoes that also vector several zoonotic flaviviruses (Le Flohic et al., 2013). Despite the knowledge that mosquito saliva contains modulatory molecules that may alter flavivirus pathogenesis in the infected host (Conway et al., 2014b; Fong et al., 2018; Pingen et al., 2016; Schneider and Higgs, 2008; Thangamani et al., 2010), whether or not the deposition of viruses by infected mosquitoes has an impact on the kinetics and severity of JEV infection has not been thoroughly examined. It has been examined previously in mice (de Wispelaere et al., 2017) and ducklings (Di et al., 2020), both of which have limited roles in JE transmission, but not in mammalian species such as swine that are involved in the enzootic transmission. Japanese encephalitis pathogenesis has been investigated and characterized under laboratory conditions in several animal models such as in mice (Li et al., 2012; Mathur et al., 1983; Tsuchiya, 1968), nonhuman primates (Myint et al., 2014; Myint et al., 1999; Raengsakulrach et al., 1999), chickens (Cleton et al., 2014), ducklings (Cleton et al., 2014; Xiao et al., 2018a; Xiao et al., 2018b), and pigs (Park et al., 2018; Ricklin et al., 2016b; Yamada et al., 2004). However, the majority of the JEV pathogenesis models for the neuroinvasive disease were established using virus-only needle inoculation. Mouse models for West Nile and dengue viruses have shown that mosquito saliva can potentiate flavivirus infections and exacerbate disease symptoms (Cox et al., 2012; Moser et al., 2016; Schneider et al., 2006; Styer et al., 2011). Therefore, the exclusion of the vector and/or its salivary components in the virus infection of the vertebrate host may potentially lead to inaccurate representations of the true virulence or pathogenesis of the virus in nature.

The objective of this study was to determine the impact of mosquito salivary components on JEV infection in pigs, a species directly involved in its transmission cycle as an amplifying host. The established method of the collection and injection of mosquito salivary gland extract to mimic the delivery of mosquito saliva when feeding was used in this study (Le Coupanec et al., 2013; Moser et al., 2016; Schneider et al., 2004; Styer et al., 2011). We hypothesized that the simultaneous delivery of mosquito SGE and infectious viruses might alter the pathological outcomes caused by JEV infection in pigs. Modulation of arbovirus infections by mosquito salivary components has been primarily demonstrated in laboratory mice but requires further evaluation with other animal models. Enhanced disease symptoms have been reported in mouse models that received mosquito saliva or SGE and challenged with alphaviruses (Fong et al., 2018; Schneider et al., 2004), bunyaviruses (Edwards et al., 1998; Osorio et al., 1996), and flaviviruses (Conway et al., 2014b; Cox et al., 2012; Moser et al., 2016; Schneider et al., 2006; Styer et al., 2011). However, as discussed earlier in Chapter 1, observations in mouse models were not consistent with other small animal models. For example, challenge of hamsters (Sbrana et al., 2005) and chickens (Langevin et al., 2001) with West Nile virus and house finches with Western equine encephalitis virus (Reisen et al., 2000) through the bites of infected mosquitoes had no demonstrable differences in disease severity nor infection outcomes when compared with needle injection. Our work in Aim 2 established a model to study the modulation of JEV infections by mosquito salivary components in pigs. This is also the first study that studied the impact of mosquito saliva on flavivirus pathogenesis in a mammalian host which develops viremia and can support the enzootic transmission of flaviviruses in nature. Understanding how mosquito saliva modulates flavivirus infections in mammalian amplifying hosts has significant implications because humans play a similar role in the urban transmission of dengue, yellow fever, and Zika

viruses.

In this study, the co-injection of SGE derived from *Cx. quinquefasciatus* and JEV through the intradermal route altered the kinetics of JEV infection in domestic pigs. As illustrated in **Figure 4.1**, a total of 28 three-week-old white-line crossbreed domestic piglets were randomly allocated into four groups and intradermally inoculated with one of the following: [1] 100 µl of sterile saline (mock or control group, n = 4), [2] mixture of 50 µl of sterile saline and 50 µl of JEV JE-91 stock containing 10^7 $TCID_{50}$ (JEV-only group, n = 10), [3] 100 µl equal volume mixture of SGE (dose equivalent to 2.5 pairs of salivary glands) and sterile saline (SGE-only group, n = 4), and [4] 100 µl equal volume mixture of SGE (dose equivalent to 2.5 pairs of salivary glands) and 10^7 $TCID_{50}$ of JEV JE-91 (SGE+JEV group, n = 10). Half of the animals from each group were euthanized at day 3 or 28 post-infection to characterize the acute or convalescent phases of infection, respectively.

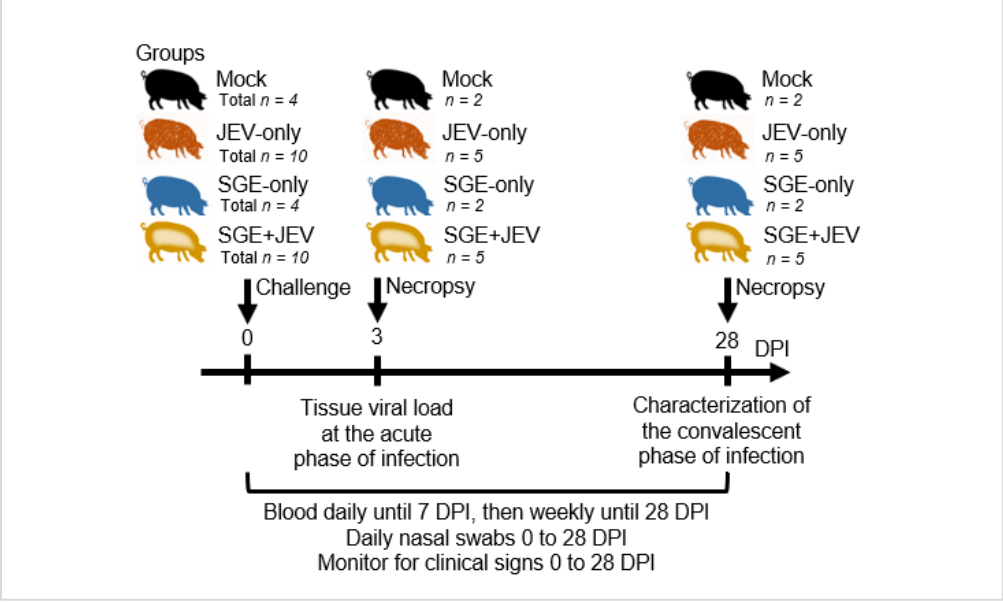

Figure 4.1. Schematic diagram of Aim 2 experimental design.
DPI = day post-infection. JEV = Japanese encephalitis virus. SGE = salivary gland extract.

In contrast to the enhancement of diseases caused by mosquito saliva reported in mouse models, SGE reduced the severity of diseases caused by JEV infection as demonstrated by the development of milder febrile illness and shortened period of viral nasal shedding. Interestingly, the viral loads among the tissues collected from the central nervous system did not differ significantly and no demonstrable effect on viremic titers were observed with the co-inoculation of SGE and JEV. The findings suggest that the modulation of flavivirus infection by mosquito saliva may result in different infection outcomes depending on the vertebrate host species.

Results

All animals were healthy and had no detectable neutralizing antibodies against JEV at the start of the study. Inoculation of co-injection of JEV and SGE or JEV alone, both led to the onset of clinical signs including fever and lethargy. Clinical signs of acute infections subsided near to the subsidence of fever or the defervescence stage followed by the development of abnormal gait changes indicating neurological damage. Kinetics in the onset of clinical signs and differences in tissue viral loads were compared to assess the impact of SGE on the kinetics and severity of JEV infection in the pigs.

Modulation of JEV-induced fever, viremia, and nasal shedding by SGE

The development of fever has been consistently observed in pigs challenged with JEV (Park et al., 2018; Ricklin et al., 2016a; Ricklin et al., 2016b; Young et al., 2020). We hypothesized that mosquito saliva could potentially modulate the onset and severity of clinical diseases induced by JEV as reflected by the observed development of febrile illness (Fong et al., 2018; Schneider and Higgs, 2008; Schneider et al., 2004). Elevated body temperatures were detected in both

SGE+JEV and JEV-only groups. One notable difference between the SGE+JEV and JEV-only groups was the time of fever onset and percentage of animals with elevated temperature, as shown in **Figure 4.2**. Injection with only JEV led to the development of fever in 10% of animals (1/10) at 1 day post-infection followed by the highest incidence of fever (80%, 4/5) observed at day 4 post-infection. The animals that received the co-injection of JEV and SGE showed delayed onset of fever and did not reach the highest incidence until day 6 after challenge (40%, 2/5). The average body temperatures of animals in the SGE+JEV group were only significantly lower than those in the JEV-only group at day 1 post-infection (Dunn-Bonferroni test, $p = 0.017$) but remained lower than those in the JEV-only group on days 2, 3, and 4 post-infection, although the comparisons were not statistically significant (Dunn-Bonferroni test, $0.155 \geq p \geq 0.999$).

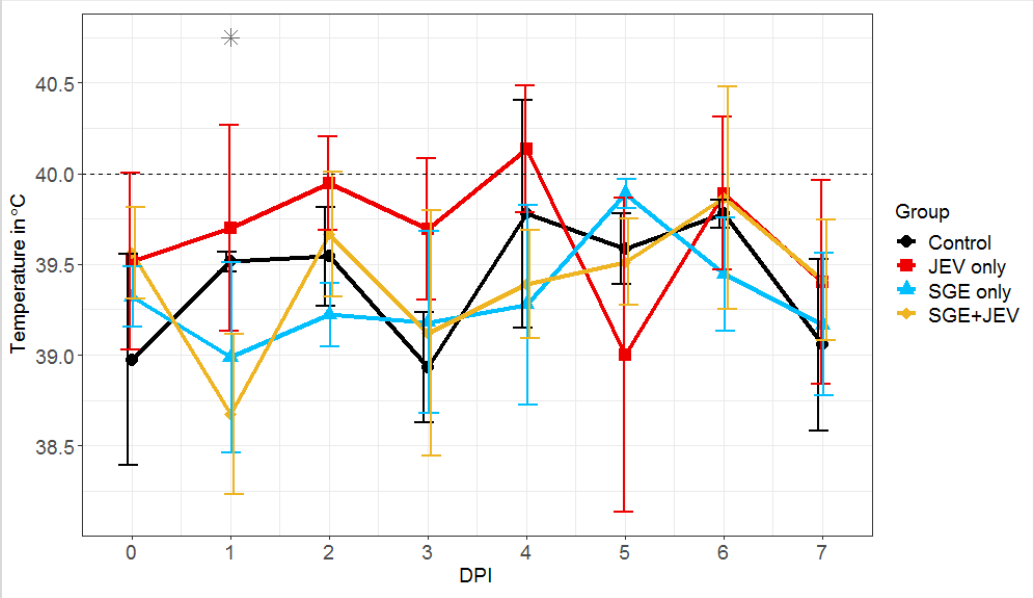

Figure 4.2. Temperatures of animals in the Aim 2 study after their respective inoculation.

Average body temperatures of pigs after intradermal challenge with sterile saline (control), 10^7 $TCID_{50}$ of JE-91 strain (JEV-only), salivary gland extract (SGE-only), and 10^7 $TCID_{50}$ of JE-91 strain mixed with salivary gland extract (SGE+JEV). DPI = day post-infection. Asterisk (*) indicates the significant difference when JEV and SGE+JEV groups were compared to each other considering time as a factor using non-parametric Kruskal-Walls test and post hoc Dunn's multiple pairwise comparison test adjusted with Bonferroni correction.

Differential kinetics in the development of febrile illness in response to the needle inoculation of JEV and the simultaneous delivery of SGE and JEV warranted the comparison of viremic and nasal shedding profiles, two important manifestations caused by JEV infection in pigs (García-Nicolás et al., 2017; Park et al., 2018; Ricklin et al., 2016a). All virus-challenged animals developed transient viremia, as shown in **Figures 4.3A** and **4.3B**. The average viremic titers and serum viral loads among the SGE+JEV animals appeared slightly lower than the JEV-only group, but the differences were not statistically significant at any time point (Dunn-Bonferroni test, $p \geq 0.999$). The highest average viremic titer observed in both groups were comparable to each other (SGE+JEV at day 2 post-infection: $2.2x10^4 \pm 3.3x10^4$ PFU/ml; JEV-only at day 3 post-infection: $1.1x10^5 \pm 2.9x10^5$ PFU/ml; Dunn-Bonferroni test, $p = 0.396$) followed by the clearance of viremia at either 4 or 5 days after challenge. Data analyzed by plaque assay was consistent with those obtained through RT-qPCR. The serum viral loads also peaked in SGE+JEV and JEV-only groups at 2 ($4.7x10^4$ geq-$TCID_{50}$/ml) and 3 days post-infection ($4.4x10^5$ geq-$TCID_{50}$/ml), respectively. No difference was found in the serum viral loads between the two groups of animals at day 2 or 3 post-infection (Dunn-Bonferroni test, $p \geq 0.999$).

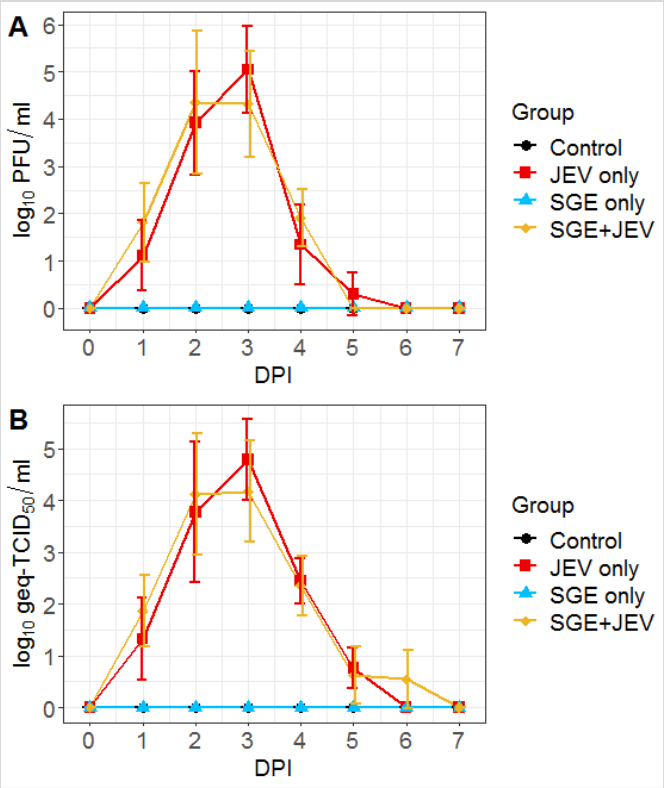

Figure 4.3. Magnitude and duration of viremia of Aim 2 animals post-challenge.

Viremic profiles of individual animals following intradermal JEV challenge with or without SGE quantified by plaque assay (A) and RT-qPCR (B). DPI = day post-infection. PFU = plaque forming units. Geq-$TCID_{50}$ = genome equivalent-50% tissue culture infectious dose.

Infectious viruses were isolated in the nasal swabs collected from the experimentally challenged pigs, as demonstrated in **Figure 4.4A**. Forty percent (4/10) of animals in the SGE+JEV group began to secrete infectious viruses as early as day 2 post-infection. Similarly, infectious virus was detected in nasal secretions collected from 60% (6/10) of pigs in the JEV-only group. Detection of infectious viruses persisted in both the SGE+JEV and JEV-only groups for up to day 6 and 5 after infection, respectively. Detection of viral RNA demonstrated that 80% (8/10) of SGE+JEV pigs and 100% (10/10) of JEV-only pigs developed nasal shedding (**Figure 4.4B**). The duration of nasal shedding was prolonged in comparison with the durations of fever and viremia. Viral RNA was detected from 2 to 7 days post-infection in the SGE+JEV group whereas nasal

shedding was detected up to day 10 after challenge in the JEV-only group. Nasal secretions had no demonstrable difference in infectious titers and viral RNA loads at any time point between SGE+JEV and JEV-only groups (Kruskal-Wallis test, $p = 0.113$ in infectious titers and Dunn-Bonferroni test, $p \geq 0.999$ in viral RNA loads). However, the average duration of nasal shedding was significantly shorter among SGE+JEV pigs (1.8 ± 1.3 days) than those challenged with JEV only (3.8 ± 1.6 days) (one-tailed *t*-test, $t = 1.925$, $p = 0.045$).

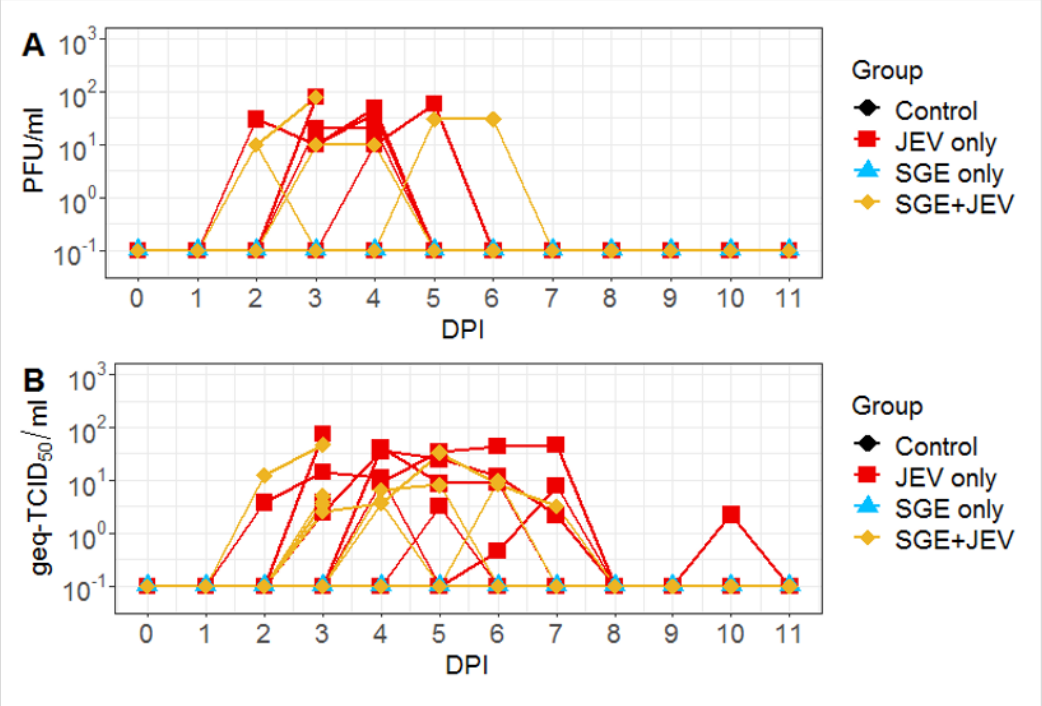

Figure 4.4. Nasal shedding profiles of JEV-infected animals.

Nasal shedding kinetics of individual animals following intradermal JEV challenge with or without SGE quantified by plaque assay (A) and RT-qPCR (B). PFU = plaque forming units. DPI = day post-infection. Geq-$TCID_{50}$ = genome equivalent-50% tissue culture infectious dose.

In summary, the injection of SGE and JEV modulated the kinetics of fever but not the viral titers of viremia or nasal shedding. The delayed onset of fever and shortened periods of nasal shedding suggest that the inclusion of SGE in the inocula altered the kinetics of acute disease signs

caused by JEV infection. However, the addition of SGE did not have a demonstrable impact in quantities of infectious viruses and viral genomes in serum and nasal secretions.

Impact of SGE on the viral burdens of different tissues

Detectable viremia led to the dispersal of JEV to lymphoid and nervous tissues through the hematogenous route in both SGE+JEV and JEV-only groups at day 3 post-infection, as detailed in **Figure 4.5**.

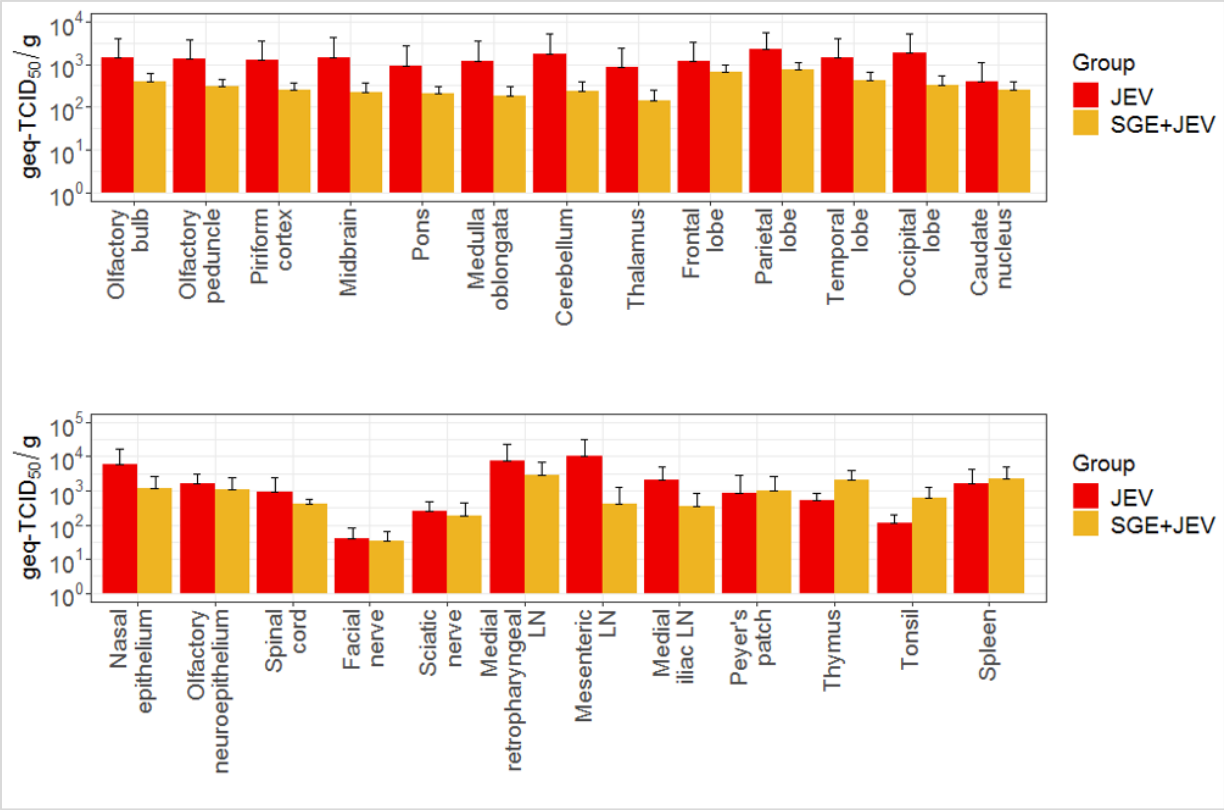

Figure 4.5. Tissue dissemination pattern of JEV in the presence of SGE at the inoculation site.

Average viral loads of several tissues collected at 3 DPI following intradermal JEV challenge with or without SGE. DPI = days post-infection. Geq-$TCID_{50}$ = genome equivalent-50% tissue culture infectious dose.

Consistent with the comparable viremia titers, the systemic spread of JEV was not impacted by the co-administration of SGE and JEV because the infectious titers and viral RNA loads of homogenized lymphoid tissues (peripheral lymphoid nodes, thymus, and tonsil) did not show demonstratable differences (Dunn-Bonferroni test, $p \geq 0.999$ in infectious titers and Kruskal-Wallis test, $p = 0.363$ for viral load) (**Figure 4.5**). The co-injection of JEV and SGE did, however, lead to different outcomes of JEV infection in one of the two peripheral nervous tissues examined in this study, as shown in **Figure 4.6**. Homogenized sciatic nerves obtained from SGE+JEV animals ($2.2x10^1 \pm 4.4x10^1$ PFU/g) had significantly lower amount of infectious viruses than those that were injected with JEV alone ($2.5x10^3 \pm 4.7x10^3$ PFU/g, Mann-Whitney U test, $p = 0.032$). However, there was no demonstrable statistical difference in the viral RNA load of the sciatic nerve samples (Mann-Whitney U test, $p = 0.548$) (**Figure 4.5**). Albeit the lack of statistical significance (Mann-Whitney U tests, $0.222 \geq p \geq 0.999$), the average viral RNA loads in the CNS collected from the SGE+JEV group were overall lower than those from the animals that were injected with JEV alone (**Figure 4.5**). In summary, SGE altered the viral burden in peripheral nervous tissues but had no demonstrable impact on the infection outcomes of lymphoid and central nervous tissues.

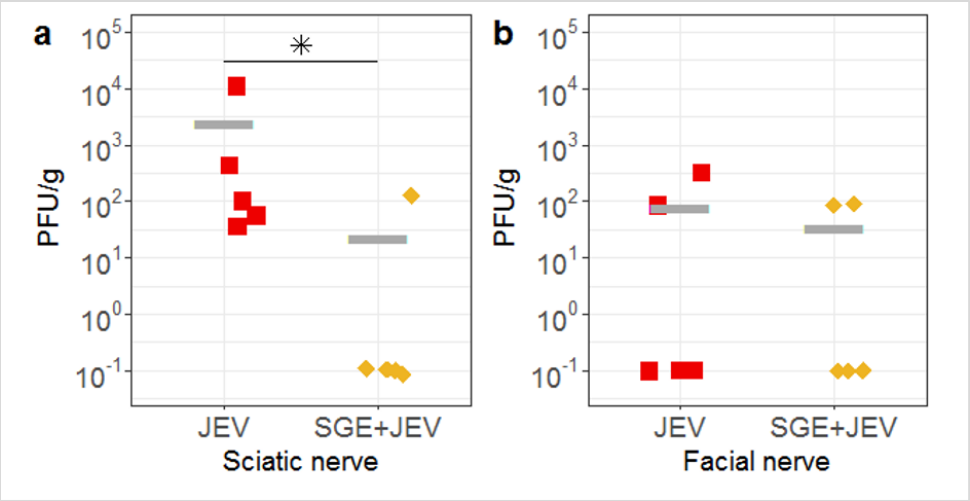

Figure 4.6. Infectious viral titers of sciatic and facial nerve samples collected from the infected animals.

Asterisk (*) indicates the significant difference when JEV and SGE+JEV groups were compared to each other using non-parametric Mann-Whitney U test.

Neurologic signs and persistent infection in pigs

Development of trembling, paralysis, and/or ataxia of the hind limbs has been previously reported in JEV-infected swine (Fujisaki, 1975; Kodama et al., 1968; Park et al., 2018; Yamada et al., 2004; Yamada et al., 2009). Animals in the SGE+JEV and JEV-only groups had a comparable incidence of ataxia (SGE+JEV: 80% (4/5); JEV-only: 40% (2/5); Fisher's exact test, $p = 0.110$). The development of ataxia in both groups is summarized in **Table 4.1**. In the SGE+JEV group, two animals became ataxic in their hind limbs as early as day 6 post-infection and persisted until the end of the study. Two additional pigs developed bilateral hind limb ataxia: one at day 11 post-infection that lasted a week and the other at day 25 after infection that was persistent until the end of study. In the JEV-only group, one pig developed gait abnormality at day 15 post-infection until the end of the study, while another exhibited only a 2-day period of mild rear limb ataxia between day 22 and 23 after challenge. Although the onset of ataxia between the two groups were statistically not different (Mann-Whitney U test, $p = 0.087$), there appeared to be a trend for earlier

onset of hind limb ataxia in the SGE+JEV group than the JEV-only group. Despite the apparent signs of neurologic abnormalities, all animals survived the experimental challenge and developed neutralizing antibody responses. Geometric mean $PRNT_{50}$ titers at day 28 post-infection were similar between the SGE+JEV (105.6) and JEV-only (91.9) groups (Mann-Whitney U test, p = 0.841).

Table 4.1. Timetable for the onset of ataxia in the experimental groups of Aim 2.

ID	Group	D0	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16	D17	D18	D19	D20	D21	D22	D23	D24	D25	D26	D27	D28
8	Control																													
9	Control																													
10	JEV only																													
11	JEV only																													
12	JEV only																A	A	A	A	A	A	A	A	A	A	A	A	A	A
13	JEV only																							A	A					
14	JEV only																													
22	SGE only																													
23	SGE only																													
24	SGE+JEV							A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
25	SGE+JEV																										A	A	A	A
26	SGE+JEV							A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
27	SGE+JEV												A		A	A	A	A	A											
28	SGE+JEV																													

ID = pig identification number. D = day. JEV = Japanese encephalitis virus. SGE = salivary gland extract. A (in green box) = ataxia.

Neurological signs observed during the convalescent phase of JEV infection were consistent with the presence of viral RNA in CNS and lymphoid tissues in both groups of animals. Viral RNA was detected in at least one CNS tissue in two animals in the SGE+JEV group and one animal in the JEV-only group at day 28 post-infection (**Figure 4.7**).

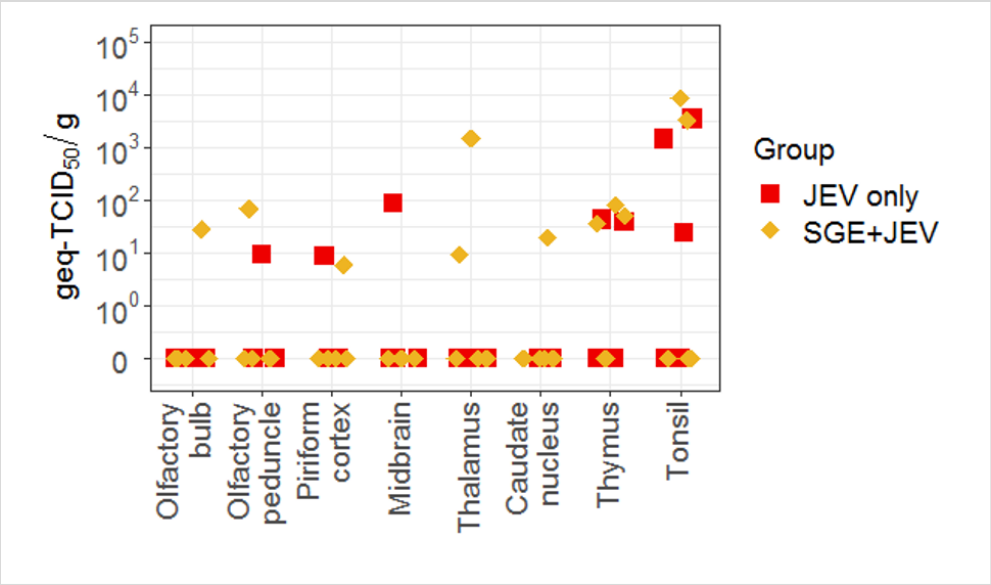

Figure 4.7. Persistent JEV infection of several nervous and lymphoid tissues.

Viral RNA detected by RT-qPCR in several central nervous and lymphoid tissues collected at 28 DPI following intradermal JEV challenge with or without SGE. DPI = days post-infection. Geq-$TCID_{50}$ = genome equivalent-50% tissue culture infectious dose.

In addition to the detection of viral RNA in CNS tissues at 28 days after challenge, thymus and tonsil were potential sites of persistent infection. Viral loads in the thymus collected from infected pigs ranged between $3.6x10^1$ to $8.12x10^1$ geq-TID_{50}/g in the SGE+JEV group (n = 3) and between $3.85x10^1$ to $4.23x10^1$ geq-$TCID_{50}$/g in the JEV-only group (n = 2). Tonsils had a higher level of viral loads as shown with average viral titers of $5.8x10^3 \pm 2.6x10^3$ geq-$TCID_{50}$/g from two SGE+JEV pigs and $1.6x10^3 \pm 1.4x10^3$ geq-$TCID_{50}$/g from three JEV-only pigs. However, there was no statistical difference across tissue samples collected at day 28 post-infection between the groups (Kruskal-Wallis test, p = 0.378), suggesting that SGE had no impact on the persistence of JEV infection in pigs.

In summary, the impact of mosquito SGE on the kinetics and severity of diseases was limited to the acute phase of JEV infection. In comparison with pigs inoculated with JEV only the co-injection of SGE with JEV led to milder diseases based on the delayed onset of fever, shortened

nasal shedding, and slightly reduced CNS viral loads. However, the reduced severity of acute JEV infection in the SGE+JEV group had no demonstrable impact on the frequency of neurological diseases and persistent infection.

Discussion

Mosquitoes play an integral role in the transmission of arboviruses. Mosquito salivary components have been increasingly recognized as an important factor that modulates vertebrate immune responses and, as a consequence, disease pathogenesis caused by arbovirus infections. Several studies suggested that mosquito salivary components delivered through feeding or injection suppress antiviral immunity and enhance pathological outcomes in mouse models. The immunosuppressive effects of mosquito saliva include the stimulation of anti-inflammatory cytokines (Schneider et al., 2010; Schneider et al., 2004; Vogt et al., 2018), polarization from a Th1 to Th2 immune response (Schneider et al., 2004; Thangamani et al., 2010), and suppression of the host innate immune responses (Fong et al., 2018; Schneider and Higgs, 2008; Thangamani et al., 2010). However, the enhancement of pathological outcomes caused by arbovirus infections has not been consistently observed in all vertebrate species (Langevin et al., 2001; Reisen et al., 2000; Sbrana et al., 2005). Our study investigated the impact of mosquito SGE on the kinetics and severity of JEV infection in pigs, an amplifying host that is directly relevant to JEV transmission in nature. This model system is unique from the majority of previously published studies because it assesses the impact of mosquito salivary components on flavivirus infections in a mammalian amplifying host.

The co-injection of SGE and JEV, an established approach developed to mimic the bite of infected mosquitoes (Le Coupanec et al., 2013; Moser et al., 2016; Schneider et al., 2004; Styer et

al., 2011), showed that mosquito salivary components can modulate JEV infection in pigs, resulting in reduced fever and decreased nasal shedding duration. Consistent with the hypothesis that mosquito saliva suppresses the pro-inflammatory responses in the vertebrate host, the simultaneous delivery of SGE and JEV led to a low incidence and delayed onset of fever, as shown in **Figure 4.2**. This effect has also been demonstrated after mosquito bite infection of a humanized dengue virus mouse model, which was partially reconstituted with human immune cells to recapitulate dengue pathogenesis in humans (Cox et al., 2012). It is also consistent with the anti-inflammatory properties of *Cx. pipiens* and *Cx. quinquefasciatus* saliva reported in human keratinocytes (Garcia et al., 2018; Zeidner et al., 1999). Although the mechanism of how mosquito saliva caused the observed shortened duration of JEV shedding remains unclear (**Figure 4.4A and 4.4B**), lower levels of viral shedding of West Nile virus has also been documented in two independent studies that compared chickens challenged via needle inoculation with those infected by mosquito bites (Langevin et al., 2001; Styer et al., 2006). Cloacal shedding of West Nile virus was less frequently detected in chickens inoculated by infected *Cx. tritaeniorhynchus* bites than chickens infected by subcutaneous injection (Langevin et al., 2001). More efficient viral clearance and shortened periods of oral shedding was also reported in chicks exposed to West Nile virus from infected *Cx. pipiens* than those infected parenterally (Styer et al., 2006). However, this trend was only documented among chickens within a specific range of age (Styer et al., 2006), suggesting that reduced shedding of flaviviruses in avian hosts is also likely to be age-specific. Nevertheless, the shortened period of nasal shedding challenges the epidemiologic importance of vector-free JEV transmission (Ricklin et al., 2016a). To date, infectious viruses and JEV genome in nasal secretions has only been detected under laboratory conditions. The existence of direct pig-to-pig transmission under field conditions has been further supported using mathematical

modelling consistent with swine serological data collected from Cambodia, a country with high JE incidence (Diallo et al., 2018). However, to the best of our knowledge, there has been no virus isolate or detection of viral genome reported in naturally infected animals, warrants further investigation in understanding the degree of importance of nasal shedding of JEV among domestic pigs and whether or not it can serve as a mechanism for viral maintenance in nature.

Our results are comparable with published studies that showed that acute diseases caused by flavivirus infections can be modulated by mosquito salivary components (Cox et al., 2012; Moser et al., 2016; Styer et al., 2011), but how the disease was impacted and altered was different. Feeding by infected mosquitoes or simultaneous injection of mosquito SGE with infectious viruses was implicated to enhance systemic diseases caused by flavivirus infections as observed previously, such as with inbred mouse strains challenged with West Nile virus (Moser et al., 2016; Styer et al., 2011) and humanized mice challenged with dengue virus serotype 2 (Cox et al., 2012). Intriguingly, the enhancement of viremia and systemic disease was not observed in our pig model. The viremic titers and serum viral loads between the SGE+JEV and JEV-only groups remained comparable, as summarized in **Figures 4.3A and 4.3B**. Additionally, consistent with the hematogenous route of neuroinvasion by JEV (Mathur et al., 1992; Monath et al., 1983; Myint et al., 2014; Nagata et al., 2015), the unaltered viremia was coupled with viral burdens in the central nervous tissues that were comparable between the animals in the SGE+JEV and JEV-only groups (**Figure 4.5**). At the same time, the incidence and severity of febrile illness was reduced among animals that received the intradermal injection of SGE and JEV (**Figure 4.2**). One explanation to these observations may be due to the differences in the choice of vertebrate species used in the studies. The seemingly contradictory outcomes may reflect the different roles of incidental and amplifying hosts in flavivirus transmission. Although a useful laboratory model, mice and other

rodent species have no known role in the transmission or maintenance of flaviviruses in nature. The development of neurotropic diseases is an important pathological outcome for most mouse models after experimental challenges with flaviviruses, which is a hallmark that resembles the incidental hosts for JEV and other flaviviruses. On the other hand, as an amplifying host species, domestic pigs develop viremia to sustain the transmission of JEV. Therefore, it is speculated that the modulation of flavivirus infections by mosquito salivary components can be fundamentally different in amplifying hosts, especially mammalian species, and in incidental hosts. Despite the limited numbers of amplifying hosts that can be studied under laboratory conditions, understanding the differential immunomodulatory outcomes by salivary components of mosquitoes may provide an opportunity to investigate how saliva of hematophagous arthropods can affect the transmission efficiency of flaviviruses in nature.

With this in mind, the lack of differences in viremic titers and serum viral loads could indicate that the modulation of disease severity by mosquito saliva in amplification hosts potentially does not significantly affect the likelihood of transmission via the bite of infected mosquitoes. However, this hypothesis requires further investigation. Amplifying hosts that develop viremia, but experience no apparent signs of disease, have been recently recognized to be advantageous for the transmission and maintenance of flaviviruses, as observed with dengue virus (Duong et al., 2015; Ten Bosch et al., 2018) and Zika virus infections in humans (Moghadas et al., 2017). In a study with dengue patients from Cambodia, infected people with no symptoms or prior to the onset of clinical illness were significantly more infectious to *Ae. aegypti* mosquitoes than the patients with symptomatic infection despite the lower average level of viremia in the asymptomatic individuals (Duong et al., 2015). As a result, the strong immune response and high cytokine production associated with clinical illness in the infected host have been proposed to play

a factor in the observed reduction of infectivity to mosquitoes (Duong et al., 2015; Rathakrishnan et al., 2012). Based on mathematical modelling analysis of host-viral dynamics and empirical data, Ten Bosch et al. (2018) therefore suggested that the asymptomatic individuals with inapparent dengue symptoms not detected by the surveillance systems may actually be the primary reservoir of dengue virus transmission. In addition, Reinhold et al. (2021) proposed that mosquitoes may possibly prefer bloodmeals from body extremities with slightly cooler temperatures due to thermal stress and energy cost associated with the ingestion of warm blood meals (Benoit et al., 2011; Lahondère and Lazzari, 2012). If supported, this could suggest that the preference by mosquitoes for bloodmeals from animals with normothermic or nonfebrile temperatures could be likely. In a thermotaxis study with *Ae. aegypti* mosquitoes, Corfas and Vosshall (2015) demonstrated that host-seeking mosquitoes are most attracted to temperature stimuli close to host body temperatures (i.e. range of 37 °C of humans to 43 °C of birds), peaking at maximum attraction at 40 °C which is considered the minimum temperature for fever (≥ 40 °C) in most domestic mammalian species (Robertshaw, 2004). How clinical illness and symptoms affect mosquito behavior in association with virus transmission still requires further investigation.

In addition to the potential variation based on the vertebrate host, the effect of mosquito saliva in the animal models may be virus-specific. Our understanding of how mosquito saliva modulates encephalitic flavivirus infections has been largely derived from West Nile virus mouse models (Moser et al., 2016; Schneider et al., 2007; Schneider et al., 2010; Schneider et al., 2006; Styer et al., 2011). In the available West Nile virus infection models, it has been implicated that mosquito saliva enhances the systemic infection followed by the development of more severe neuroinvasive diseases. However, our work and that of others suggest that mosquito saliva may potentially play a different role in modulating the outcomes of JEV infections in the vertebrate

host, but the results are variable and inconsistent from one another. For example, the co-injection of SGE or saliva collected from *Cx. pipiens* or *Ae. albopictus* and JEV had no demonstrable impact in the viremia and mortality of BALB/c mice in comparison with intradermal inoculation of JEV alone (de Wispelaere et al., 2017). In contrast, high viremia and high mortality from viral encephalitis with apparent neurological signs were induced by bites from JEV-infected *Cx. pipiens* in newborn ducklings (Di et al., 2020), which normally do not demonstrate such clinical outcomes to JEV by needle inoculation (Xiao et al., 2018b). These variations in infection outcomes again could most likely be due to the differences in the disease pathogenesis of JEV in different vertebrate species.

These inconsistent results could also potentially be due to differences in experimental methodology**,** such as the use of SGE to emulate mosquito saliva or bite. However, the effects of SGE and mosquito saliva or bite have been proven to create similar effects in the vertebrate host (Le Coupanec et al., 2013; Moser et al., 2016; Schneider et al., 2004; Styer et al., 2011) and virus replication in the vector has been shown to not be necessary for the disease enhancement effects to be observed (Edwards et al., 1998; Styer et al., 2011). Moser et al. (2016) showed that as little as 0.01 μg of SGE protein, which is approximately equivalent to 0.01 salivary gland pairs, could cause enhancement of West Nile virus infection in mice and dose-dependent enhancement occurred with increasing concentration of SGE or number of mosquitoes spot-feeding at the inoculation site.

Additionally, the effects of mosquito saliva could be mosquito species-dependent. For example, while mortality rates significantly increased from Rift Valley fever virus when SGE from *Ae. vexans* and *Ae. aegypti* were used, SGE from *Cx. pipiens*, although a competent vector of Rift Valley fever virus, did not produce any observable effects on mice survival (Le Coupanec et al.,

2013). Vector competence was also considered not an important factor in another study, in which outbred mice resistant to infection of Cache Valley virus by subcutaneous injection became viremic and developed antibodies when the virus was injected into sites that were spot-fed by mosquito species that are not natural vectors of the virus, such as *Ae. triseriatus*, *Ae. aegypti*, and *Cx. pipiens* (Edwards et al., 1998). To complicate this further, other factor may be involved in how mosquito saliva may impact virus infection because mosquito saliva or certain SGE fractions from *Ae. aegypti* caused reduction of dengue virus infectivity *in vitro* (Ader et al., 2004; Conway et al., 2014a; Conway et al., 2014b), but enhancement of dengue disease using the same mosquito species was reported in another study (Cox et al., 2012; Huang et al., 2019). In our study, the use of *Culex* species mosquito that are involved in natural transmission of JEV (Weng et al., 1999) ensures the biological relevance of the model developed to study the modulation of JEV infections by mosquito saliva. Ultimately, the different results may be due to the inherent differences in the interactions among the mosquito, virus, and vertebrate host such that effects of mosquito saliva may vary depending on the source, type, and species of the players involved in the arbovirus transmission. Differences in our and others' observations highlight the complexity of interactions among mosquitoes, JEV, and vertebrate hosts.

Collectively, our study demonstrates for the first time the utility of pigs to study the modulation of JEV infection by mosquito saliva. Our findings further highlight the complex and unique differences involved in the mosquito-virus-host interactions. Investigating the mechanisms responsible for these differences may be of importance to improve our understanding of the ecology and pathogenesis of arboviruses to develop the appropriate risk mitigation strategies and effective countermeasures for their transmission and disease.

Chapter 5 - North American feral swine model for JEV infection and pathogenesis

The studies from Aims 1 and 2 demonstrated the susceptibility of North American domestic pigs to JEV and characterized their infection and disease outcomes. While wild or feral pigs are thought to be important in the enzootic transmission of JEV, no direct challenge studies have been conducted to support this statement. The objective of Aim 3 was to determine the susceptibility of feral pigs to JEV and characterize the infection outcome, using the Sinclair miniature research swine breed as a feral pig representative of North America. The Sinclair miniature pig, also known as the Minnesota or Hormel miniature pig, is an established research colony developed by crossbreeding four feral pig strains found in the United States (McAnulty et al., 2011; Schook and Tumbleson, 1996). This model was used because hunting, transporting, and possessing feral pigs is illegal in Kansas (2020; Bevins et al., 2014). The study will test the working hypothesis that feral pigs are capable of developing viremia and similar pathologic outcomes observed in domestic pigs. Results from this animal study were compared with those obtained from the domestic pigs in the JEV-only group in Aim 2. The work displayed here in this chapter will be prepared for future submission for peer review and publication.

Introduction

Wild boars or feral pigs are becoming increasingly recognized to have high

epidemiological importance in the transmission or maintenance of several zoonotic pathogens including viruses, such as hepatitis E virus and influenza virus, and bacterial enteropathogens (Meng et al., 2009; Miller et al., 2017; Petersen et al., 2020; Ruiz-Fons, 2017). By definition, wild or feral boars are a group of *Sus scrofa* biotypes that includes feral or escaped domestic pigs, Eurasian or Russian wild boars, and their cross-bred hybrids (Ruiz-Fons, 2017). A list of the pathogens with public health significance most likely to be transmitted from the wild or feral pig population to humans are provided in **Table 5.1**. Feral pigs are also reservoirs for pathogens important to the domestic swine industry, such as pseudorabies virus, *Brucella suis*, and porcine reproductive and respiratory syndrome virus (Wyckoff et al., 2009). The importance of feral pigs for these diseases primarily rises from the concern that they may serve as an unmonitored pathogen source of potential spillover to the livestock and human population (Petersen et al., 2020; Pierce et al., 2020). Potential routes of exposure to pathogens from wild pigs include consumption of their undercooked or raw meat products, direct contact, indirect contact via their interaction with livestock and companion animals, handling of their carcass, consumption of contaminated food and water, and bites from arthropod vectors (Ruiz-Fons, 2017). For example, there has been a case report of hepatitis E virus transmission to human in Japan from the consumption of infected wild boar meat (Li et al., 2005). Feral pigs have been implicated to be involved in various brucellosis outbreaks in the United States affecting a cattle herd, several domestic swine farms, and subsequently humans (Glazier, 2017; Wyckoff et al., 2009). Additionally, it is estimated that up to 60% of the several outbreaks of classical swine fever that affected the domestic pigs in Germany in the 1990s were due to direct or indirect contact with infected wild boars (Moennig, 2015).

These cross-species disease transmission events are primarily due to the increasing interaction among wild swine, domestic livestock, and humans as a consequence to the growing

population and expanding geographic distribution of wild pigs (Miller et al., 2017). In the United States, a dramatic increase in the feral pig population has been recorded over the past 30 years (Bevins et al., 2014; Snow et al., 2017), as shown in **Figure 5.1**. There is also an overall increase in overlap in terms of locality of feral pigs with livestock and humans, as presented in **Figure 5.2**. Data from GPS (geographic positioning system) movement tracking have demonstrated that feral swine frequently interact with domestic swine that are kept in outdoor pens (Wyckoff et al., 2009). In fact, close interactions and co-mingling between feral pigs and domestic livestock is common in general throughout North America in areas where they allow sharing of outdoor pasture resources (Miller et al., 2017). As such, there is a growing concern that wild or feral pigs may act as a source of pathogens that could be introduced into livestock and human populations (Peper et al., 2021). With arthropod-borne diseases, a susceptible population of feral pigs could modulate vector density and possibly impact the maintenance or persistence of the pathogen in nature if they are capable of being an amplifying host (Ruiz-Fons, 2017).

Table 5.1. List of zoonotic pathogens identified as the most prone to be transmitted from wild swine to humans and/or domestic livestock.

Viruses	Bacteria	Parasites
• Hepatitis E virus • Influenza virus • Nipah virus • Japanese encephalitis virus	• *Salmonella* species • Shiga toxin-producing *Escherichia coli* • *Campylobacter* species • *Leptospira* species • *Brucella suis* • *Mycobacterium bovis*	• *Trichinella* species

References: (Meng et al., 2009; Miller et al., 2017; Petersen et al., 2020; Ruiz-Fons, 2017)

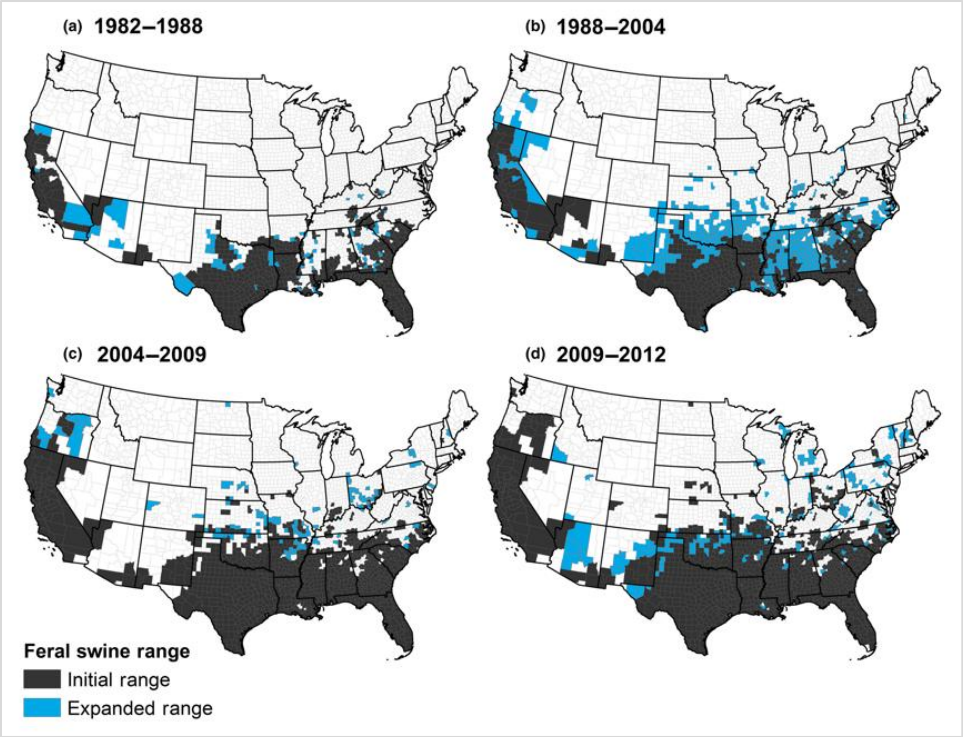

Figure 5.1. Expanding distribution of feral pigs in the United States.

Distribution data showing the spread of invasive wild pigs *Sus scrofa* throughout four time periods in the continental USA. Counties reported to be occupied by invasive wild pigs at the beginning of each time period were designated as initial range, and any other counties occupied throughout each time period were designated as expanded range. (Image taken from Snow et al. (2017); Contributed by U.S. government employees and published in the public domain).

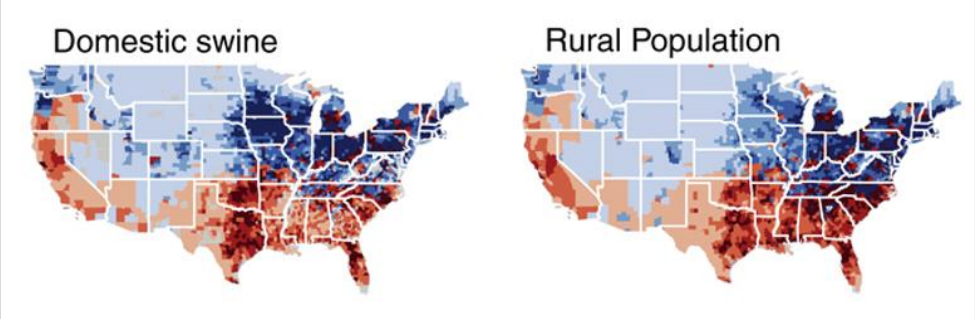

Figure 5.2. Overlapping distribution of feral pigs with those of domestic pig farms or rural human population.

County level co-occurrence of wild pigs, agricultural commodities, and rural human populations in the contiguous United States for 2012. Red shading denotes by quartile the absolute farms density (farms per km^2) or rural human population density (people per km^2) within counties co-occurring with wild pigs while blue shading indicates counties without wild pigs. (Image modified from Miller et al. (2017); Published under the Creative Commons Attribution License).

Available evidence suggests that wild boars or feral pigs may contribute to the spread and maintenance of JEV through enzootic transmission in the endemic regions. However, there have not been experimental data to validate this hypothesis due to the complicated logistics involved in procuring and handling the wild animals which may have hindered these investigations. Additionally, legislations and regulations sometimes prohibits their hunting and transport in some areas. Nevertheless, understanding the kinetics of JEV in feral pigs by experimental inoculation and infection is essential for the development and establishment of appropriate control measures for potential JE disease outbreaks. At present, available data regarding JEV in wild swine are mainly derived from JE-endemic regions based on serological studies (Hamano et al., 2007; Nidaira et al., 2014; Nidaira et al., 2007; Ohno et al., 2009; Yang et al., 2012a) with limited reports on RNA detection (Nidaira et al., 2008; Tan et al., 2012). Based on the high seroprevalence, it has been suggested that wild swine may be frequently exposed and are susceptible to JEV similar to their domestic counterparts and could be important amplifiers of JEV in nature. Accordingly, wild or feral pigs have been recognized as potential relevant drivers of JEV outbreaks in endemic areas (Hamano et al., 2007; Nidaira et al., 2014; Ruiz-Fons, 2017). Nevertheless, although epidemiological links have been suggested, the exact role of feral pigs as potential virus reservoirs and a possible source of infection is still unclear. Further studies are thereby required to establish the viral titers in feral pigs to assess their susceptibility to disease and infection by JEV.

Domestic pigs are highly susceptible to JEV infection and are important amplifying hosts of JEV in both enzootic and epizootic transmission cycles. Wild boars or feral pigs are closely related species. Therefore, it is reasonable to hypothesize that feral pigs could also act as important reservoirs of JEV in nature but direct data is needed to support this statement. The objectives of

this study were to address this gap of knowledge by determining the susceptibility of North American feral pigs to JEV, to characterize and compare the pathogenesis of JEV in feral pigs to what has been observed in domestic pigs, and to establish an alternate feral pig model for JEV.

The Sinclair miniature research pig was used as a representative model of feral pigs to accomplish Aim 3. It is an established colony developed by the Hormel Institute at the University of Minnesota by crossbreeding four feral pig strains found in the United States (i.e. Guinea hog from Alabama, wild boar from Catalina Island, Piney wood pig from Louisiana, and dwarf Ras-n-Lansa pig from Guam in the Mariana Islands) with a domestic Yorkshire boar (McAnulty et al., 2011; Schook and Tumbleson, 1996). It is the first strain of miniature pig developed and made available to scientists for research purposes (Bouchard et al., 1996). Sinclair miniature feral pigs have been used as an animal model for translational medical research in multiple field areas, such as oncology (Misfeldt and Grimm, 1994), toxicology (Brown and Hutcheson, 1973), neural development (Ryan et al., 2018), and metabolic disease (Stricker-Krongrad et al., 2016), as well as miniature models for diseases of conventional domestic pigs (Blagburn et al., 1991; Turnquist et al., 1993) due to their smaller size, ease in handling, and thus, lower cost associated with husbandry.

To the best of our knowledge, our study is the first use of Sinclair miniature pigs as a viral infection model and the first study designed to evaluate if pigs with feral genetics and phenotypes are susceptible to JEV. In this study, the Sinclair miniature pigs were intradermally inoculated with a representative strain for genotype Ib (JE-91 strain) to determine their susceptibility and characterize the disease pathogenesis. As illustrated in **Figure 5.3**, a total of 14 three-week-old Sinclair miniature piglets were randomly allocated into the following two experimental groups: mock group (n = 4) and JEV group (n = 10). The mock animals were intradermally inoculated with

100 µl of sterile saline while the animals in the JEV group received 100 µl of 10^7 $TCID_{50}$ of JEV JE-91 strain. To characterize the acute and convalescent stages of infection, groups of seven pigs (five infected and two control pigs) were sacrificed at days 3 and 28 post-infection, respectively.

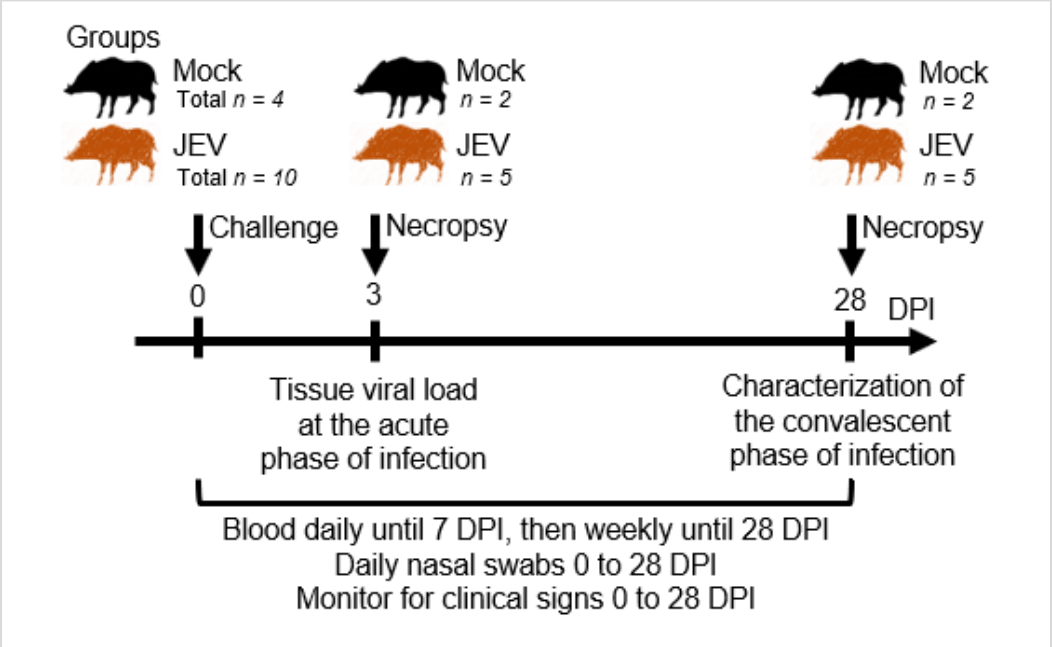

Figure 5.3. Schematic diagram of Aim 3 experimental design.
DPI = days post-infection. JEV = Japanese encephalitis virus.

Establishment of JEV infection and pathogenic outcomes were demonstrated by the detection of infectious viruses and viral genomes in multiple tissues, as previously performed for Aims 1 and 2. Intradermal JEV challenge of these pigs resulted in high viremia, viral nasal shedding, and systemic dissemination comparable to JEV infection in domestic pigs. These findings are the first direct evidence to show that feral pigs can support the enzootic transmission of JEV and highlight their potential importance in JEV transmission in nature.

Results

All animals in the study were apparently healthy and seronegative against JEV prior to the start of the experiment. Infection outcomes, such as clinical disease, viremia kinetics, and viral tissue dissemination pattern, were compared to those observed from the group of domestic pigs from Aim 2 that were intradermally challenged with JEV only.

Clinical outcomes of feral pigs infected with JEV

Fever and mild to no clinical signs are typically reported in domestic pigs infected with JEV (Park et al., 2018; Platt and Joo, 2006; Ricklin et al., 2016b). Overall, JEV infection in the Sinclair miniature feral pigs progressed similarly to what has been described in domestic pigs in terms of clinical disease. There were no overt signs of illness. Only one feral pig was depressed for two days at day 4 post-infection, but recovered to normal mentation and activity. Elevated body temperatures (≥ 40 °C) of only 1 to 2 day duration was recorded in 60% (3/5) of the feral pigs in the convalescent group. In comparison, domestic pigs from Aim 2 had a range of 1 to 6 day duration of fever in 80% (4/5) of the pigs in the corresponding group. As presented in **Figure 5.4** and **Table 5.2**, the average temperature peak in the feral pigs (39.69±0.42 °C) occurred at day 4 post-infection, in which 40% (2/5) of the feral pigs had elevated body temperatures of greater than 40°C. The average temperature peak also occurred at day 4 post-infection for JEV-infected domestic pigs, reaching a mean of 40.13±0.35 °C in which 80% (4/5) of the domestic pigs were febrile. Despite some of these differences, there was no statistical significance in the peak temperatures nor incidence of fever between the feral and domestic pigs at day 4 post-infection (Dunn-Bonferroni test, $p \geq 0.999$ for temperature and one-tailed Fisher's exact test, $p = 0.500$ for fever incidence). Overall, the body temperatures of the control pigs from the domestic group

appeared higher than those in the feral group, but there was also no demonstrable statistical difference between the two control groups (Dunn-Bonferroni test, $0.938 \leq p \leq 0.999$). The range of the average temperatures from day 0 to day 7 post-challenge was 38.66 °C to 39.36 °C and 38.51 °C to 39.77 °C for the non-infected feral and domestic pigs, respectively.

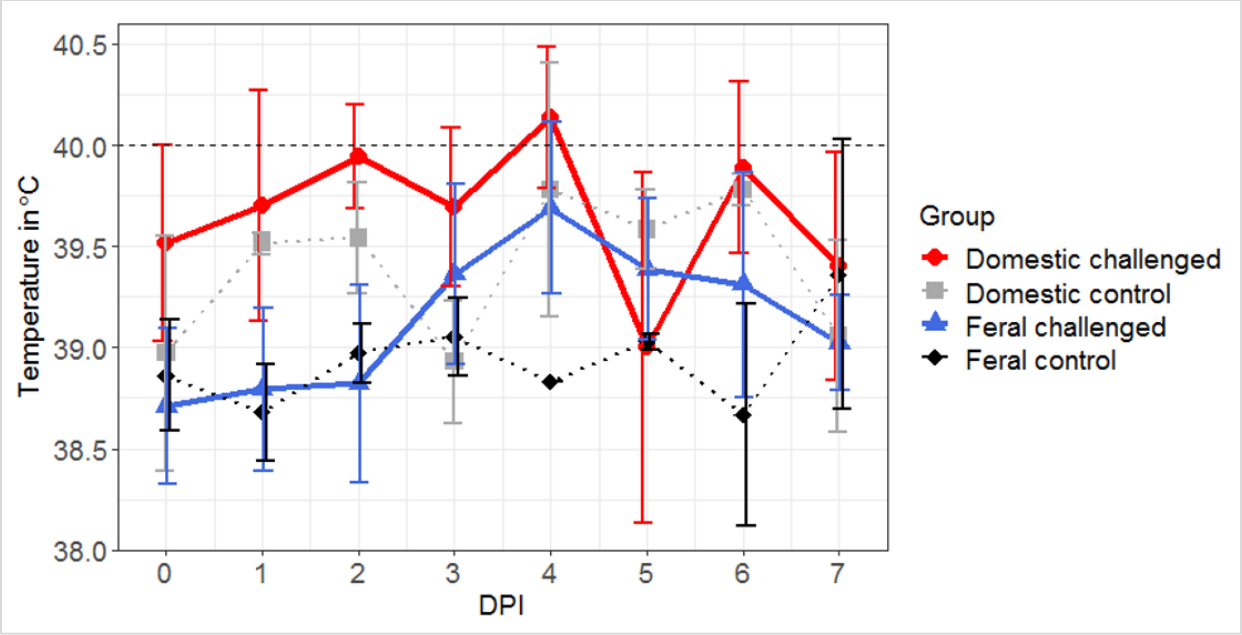

Figure 5.4. Temperature profiles of feral pigs after JEV challenge.

Body temperatures recorded from the non-infected and infected feral pigs were compared to those collected from the domestic pigs in Aim 2. DPI = day post-infection.

Table 5.2. Number of JEV-infected animals with fever (≥ 40 °C).

Group	1 DPI	2 DPI	3 DPI	4 DPI	5 DPI	6 DPI	7 DPI
Feral pigs (challenged ID with JEV alone)	0/10 (0%)	0/10 (0%)	1/10 (10%)	2/5 (40%)	0/5 (0%)	1/5 (20%)	0/5 (0%)
Domestic pigs (challenged ID with JEV alone)	3/10 (30%)	4/10 (40%)	1/10 (10%)	4/5 (80%)	0/5 (0%)	2/5 (40%)	0/5 (0%)

DPI = days post-infection. ID = intradermal. JEV = Japanese encephalitis virus. Yellow box = peak incidence.

Mild to moderate hindlimb ataxia was observed in some of the JEV-challenged feral pigs past the acute phase of infection, as summarized in **Table 5.3**. Four out of the five (80%) feral animals developed hindlimb ataxia around 11 to 15 days post-infection and recovered to normal ambulation in 1 to 8 days. Despite the earlier onset of ataxia in the feral pigs, the incidence and duration of ataxia were comparable to what was demonstrated with domestic pigs infected with JEV (one-tailed Fisher's exact test, p =0.262 for incidence and Mann-Whitney U test, p = 0.233 for duration). All feral pigs survived the challenge to the end of the study and developed a mean geometric $PRNT_{50}$ titer of 121.26, which was comparable to the titer (91.9) calculated for the domestic pigs (Mann-Whitney U test, p = 0.690).

Table 5.3. Timetable for the onset of ataxia in JEV-infected feral and domestic pigs.

Group	D0	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16	D17	D18	D19	D20	D21	D22	D23	D24	D25	D26	D27	D28
Domestic																													
Domestic																													
Domestic																A	A	A	A	A	A	A	A	A	A	A	A	A	A
Domestic																							A	A					
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D = day. A (in green box) = ataxia.

Viremic profiles of JEV-infected feral pigs

Intradermal inoculation with JEV led to detectable viremia of high magnitude and duration in the Sinclair miniature feral pigs similar to JEV-infected domestic pigs in Aim 2, suggesting that feral pigs are susceptible to JEV infection and can potentially play the role of amplifying hosts. The kinetics of viremia depicted by plaque assay and RT-qPCR were consistent to each other. As shown in **Figure 5.5**, viremia was detected at day 1 post-infection and lasted 4 to 5 days similarly to what was previously characterized in the experimental infection of domestic pigs. However,

viremia in the feral pigs reached its peak in both infectious titers and viral RNA loads a day earlier when compared to the domestic pigs challenged with JEV only in Aim 2. The peak mean infectious titer and viral load for the feral pigs occurred at day 2 post-infection and was $7.0x10^4 \pm 1.1x10^5$ PFU/ml and $5.6x10^4 \pm 9.7x10^4$ geq-$TCID_{50}$/ml, respectively. Despite this early viremic peak, these viral titers were not significantly different when compared to the levels detected in day 2 or day 3 (i.e. day of peak viremia) post-infection in infected domestic pigs (Dunn-Bonferroni test, $p \geq 0.999$ for both infectious titer and RNA load). The viral burden in the serum at 1 day post-challenge appeared higher in the feral pigs ($9.3x10^2 \pm 8.6x10^2$ PFU/ml or $9.0x10^2 \pm 6.0x10^2$ geq-$TCID_{50}$/ml) compared to the corresponding titers from the domestic pigs ($1.3x10^1 \pm 1.7x10^1$ PFU/ml or $6.6x10^1 \pm 7.0x10^1$ geq-$TCID_{50}$/ml), but they were also not statistically different from each other (Dunn-Bonferroni test, $p = 0.809$ for infectious titer and $p = 0.456$ for viral RNA load). Following the trend observed with the clinical signs, the kinetics of viremia were indistinguishable between the feral and domestic pigs after JEV challenge, strengthening the possibility that feral pigs can also be efficient amplifying hosts of JEV in both enzootic or epizootic transmissions.

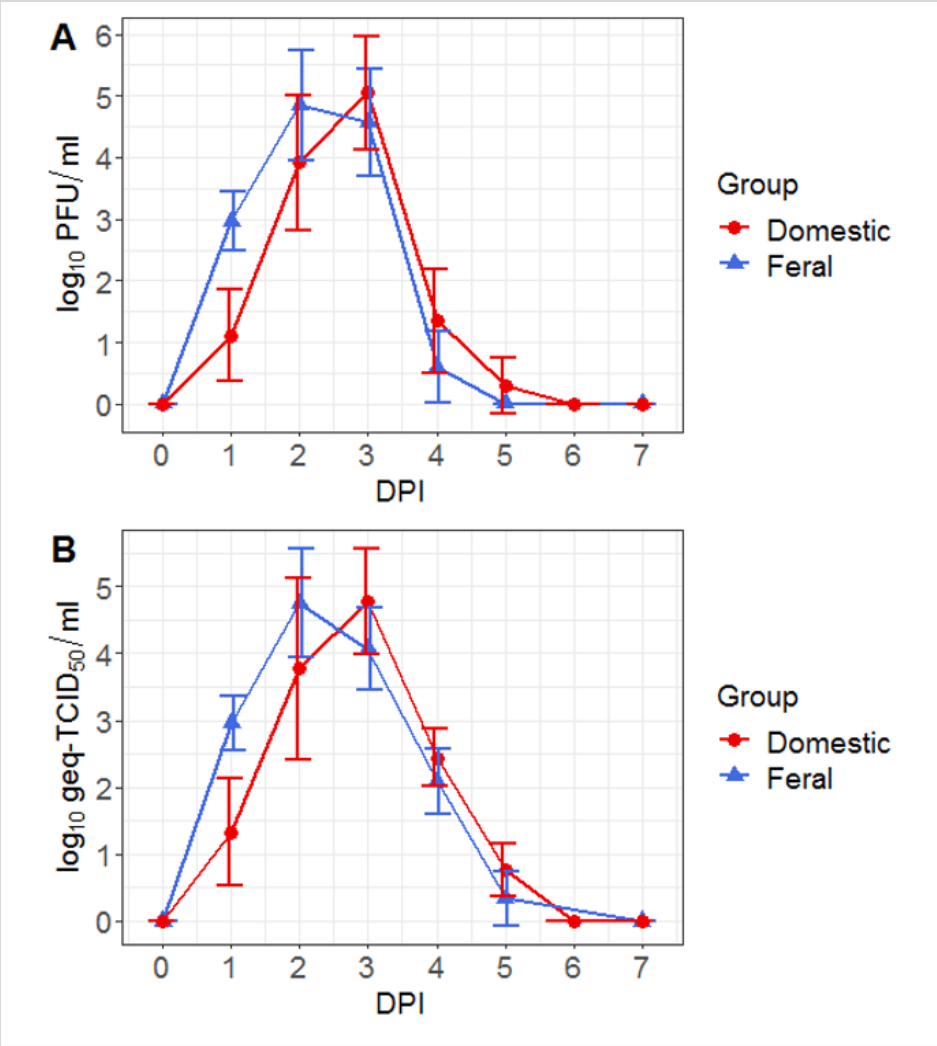

Figure 5.5. Magnitude and duration of viremia of feral pigs after JEV challenge.

Viral infectious titers (A) and RNA loads (B) detected in the serum from the infected feral pigs were compared to those obtained from the domestic pigs infected with JEV alone in Aim 2. DPI = day post-infection. PFU = plaque forming units. Geq-$TCID_{50}$ = genome equivalent-50% tissue culture infectious dose.

Kinetics of viral nasal shedding

Comparable viral burdens were detected in the nasal secretions of the Sinclair miniature feral pigs after JEV challenge to those collected from the infected domestic pigs (Dunn-Bonferroni test, $p \geq 0.999$ for infectious titers and viral RNA load), as depicted in **Figure 5.6**. In terms of infectious titers, only 40% (2/5) of the infected feral pigs shed an average of $1.8x10^1 \pm 1.0x10^1$

PFU/ml for 1 to 2 days starting at day 3 post-challenge whereas 80% (4/5) of the infected domestic pigs shed an average of $2.8x10^1 \pm 1.9x10^1$ PFU/ml for 2 to 3 days starting at day 2 after inoculation. Data analyzed with RT-qPCR was consistent with the results from the plaque assay. While the RNA loads detected at any time point were not statistically different between the two groups of animals (Dunn-Bonferroni test, $p \geq 0.999$), the duration of nasal shedding was significantly longer in the JEV-infected domestic pigs (one-tailed *t*-test, $t = -3.055$, $p = 0.008$). Infected feral pigs shed for only 1±1 days while infected domestic pigs shed for 3.8±1.8 days. For both types of pigs, the highest average RNA loads detected in the nasal swabs were on day 4 post-infection with 6.1±8.2 geq-$TCID_{50}$/ml and $1.9x10^1 \pm 1.6x10^1$ geq-$TCID_{50}$/ml in the feral and domestic pigs, respectively. The overall shedding incidence and the shedding incidence at day 4 (i.e. day of peak nasal titers) post-infection were not statistically different between the two groups of infected pigs (one-tailed Fisher's exact test, $p = 0.222$ for overall incidence and $p = 0.262$ for incidence at day 4 post-infection) (**Table 5.4**). No positive JEV detection was made after the first week post-challenge in the feral pigs.

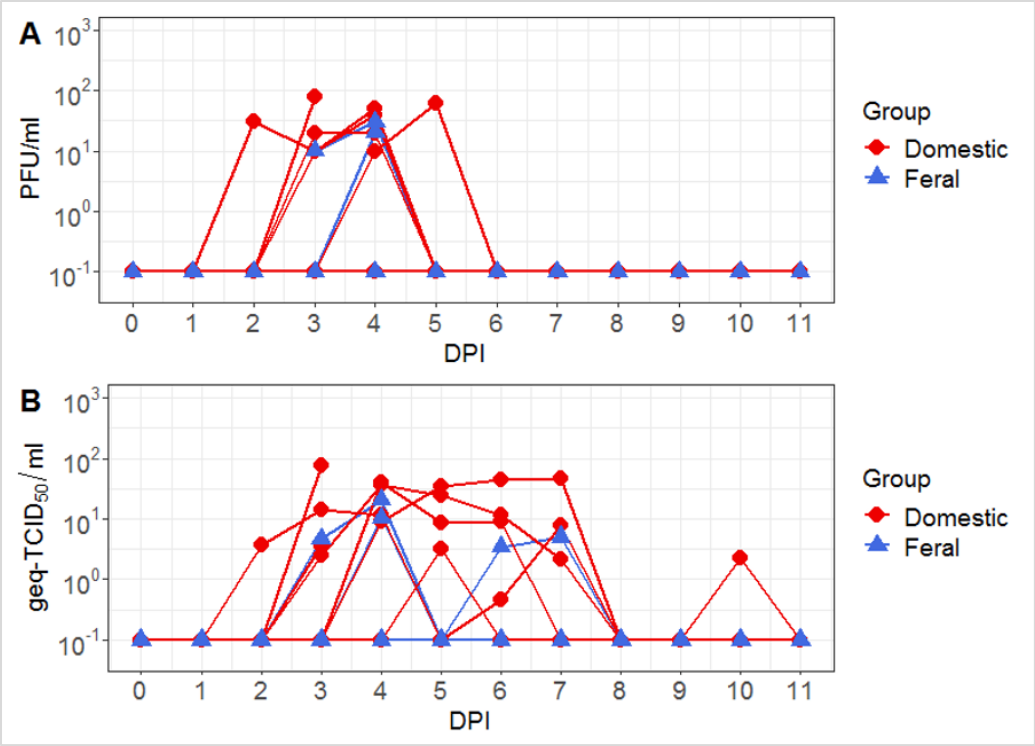

Figure 5.6. Nasal shedding of JEV by JEV-infected feral pigs.

Viral infectious titers (A) and RNA loads (B) detected in the nasal swabs from the infected feral pigs were compared to those obtained from the domestic pigs infected with JEV alone in Aim 2. DPI = day post-infection. PFU = plaque forming units. Geq-$TCID_{50}$ = genome equivalent-50% tissue culture infectious dose.

Table 5.4. Number of nasal shedders of JEV RNA in each experimental group.

Group	1 DPI	2 DPI	3 DPI	4 DPI	5 DPI	6 DPI	7 DPI
Feral pigs (challenged ID with JEV alone)	0/10 (0%)	0/10 (0%)	1/10 (10%)	2/5 (40%)	0/5 (0%)	1/5 (20%)	1/5 (20%)
Domestic pigs (challenged ID with JEV alone)	0/10 (0%)	1/10 (10%)	6/10 (60%)	4/5 (80%)	4/5 (80%)	4/5 (80%)	3/5 (60%)

DPI = days post-infection. ID = intradermal. JEV = Japanese encephalitis virus. Yellow box = peak incidence.

Viral burdens of different tissues at the acute phase of infection

Evidence of systemic dissemination of JEV at the acute phase of infection was demonstrated based on the detection of infectious viruses and JEV RNA in different tissues

sampled from the Sinclair miniature feral pigs at day 3 post-challenge. The presence of infectious viruses and viral RNA in several CNS tissues also demonstrated that JEV can lead to neuroinvasion in feral pigs similar to domestic pigs (Park et al., 2018; Ricklin et al., 2016b; Yamada et al., 2004; Young et al., 2020). Infectious titers of homogenized nervous and lymphoid tissues did not show demonstrable differences between infected feral and domestic pigs (Dunn-Bonferroni test, $p \geq 0.999$ and Mann-Whitney U tests, $0.095 \leq p \leq 0.999$), and ranged from $2.6x10^1 \pm 3.2x10^1$ PFU/g (medial iliac lymph node) to $5.4x10^3 \pm 1.0x10^4$ PFU/g (mesenteric lymph node). Analysis by RT-qPCR validated the results from the plaque assay. Consistent with the infectious titers, JEV RNA loads in the homogenized tissues were not significantly different between those sampled from the infected feral and domestic pigs (Dun-Bonferroni test, $p \geq 0.999$ and Man-Whitney U tests, $0.056 \leq p \leq 0.999$) except for piriform cortex (Mann-Whitney U test, $p = 0.016$) and occipital lobe (Mann-Whitney U test, $p = 0.016$), as shown in **Figure 5.7**. The average viral load of homogenized piriform cortex was 3.5±6.9 geq-$TCID_{50}$/g and $1.3x10^3 \pm 2.0x10^3$ geq-$TCID_{50}$/g in the feral and domestic pigs, respectively. The average viral load was also significantly lower in the occipital lobe of feral pigs ($1.9x10^1 \pm 3.7x10^1$ geq-$TCID_{50}$/g) in comparison to those from the domestic pigs ($1.8x10^3 \pm 3.1x10^3$ geq-$TCID_{50}$/g). This may suggest that while JEV is similarly neuroinvasive in the feral pig as in the domestic pig, the dissemination within the brain could potentially be slightly different. In summary, the overall tissue dissemination pattern of JEV was comparable between the feral and domestic pigs after challenge.

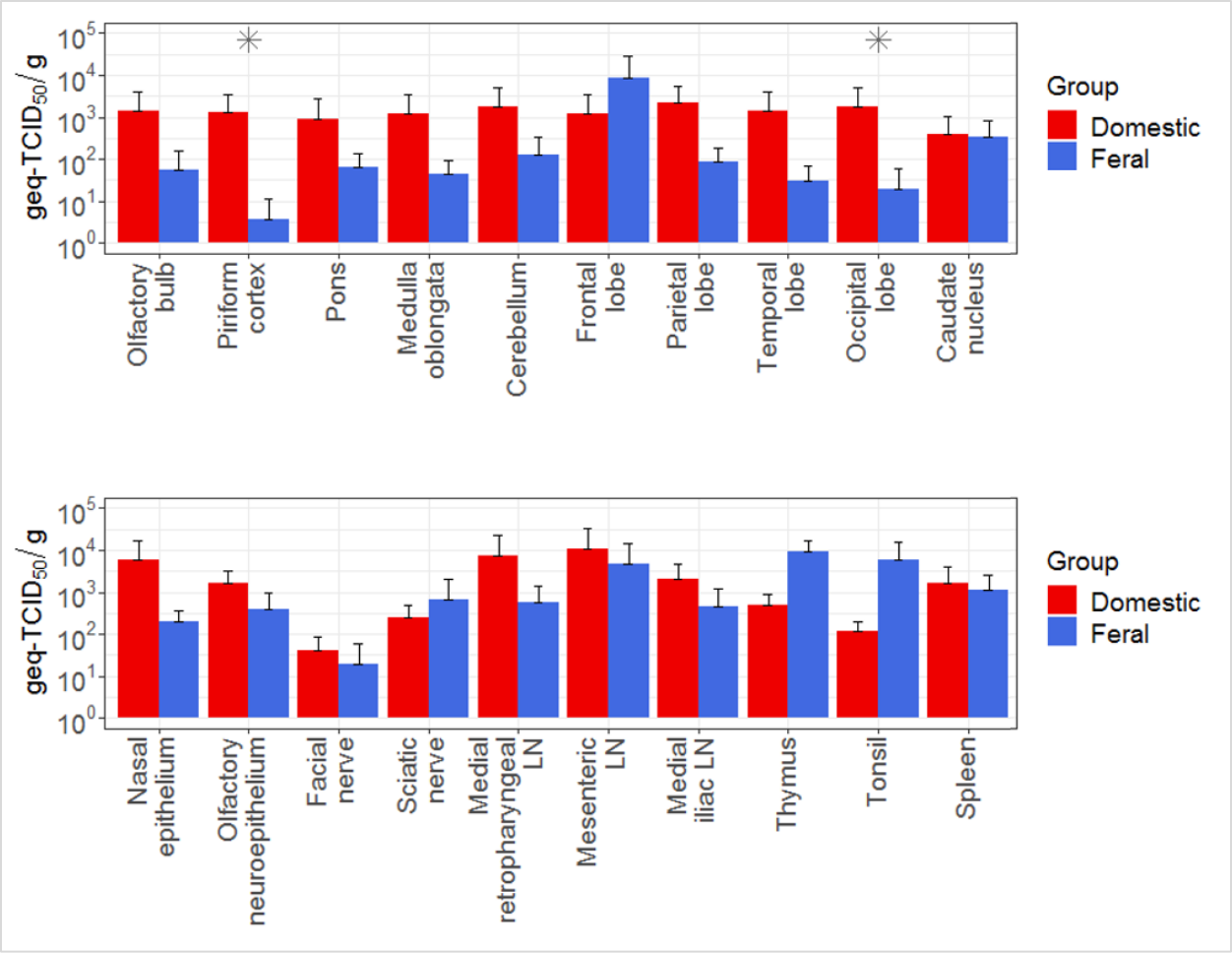

Figure 5.7. Tissue dissemination pattern of JEV in feral pigs after challenge.

Viral RNA loads detected in several different tissues collected at day 3 post-infection from the infected feral pigs were compared to those obtained from the domestic pigs infected with JEV alone in Aim 2. Geq-$TCID_{50}$ = genome equivalent-50% tissue culture infectious dose. Asterisk (*) indicates the significant difference when feral and domestic pig groups were compared to each other using non-parametric Mann-Whitney U test.

Persistent infection of JEV in feral pigs

Based on the detection of JEV RNA in tissues collected at day 28 post-infection by RT-qPCR, persistent infection of JEV was demonstrated in the Sinclair miniature feral pig, suggesting that they can also be used as an additional model of JEV persistence. In contrast to the domestic animals of the JEV-only group in Aim 2, viral RNA was not detected in the brain at 28 day post-

infection. The one pig from the JEV-only domestic group that was ataxic in the hindlimbs until the end of the study was the only animal that had persistent JEV RNA present in the brain (i.e. olfactory peduncle, piriform cortex, and midbrain) in Aim 2. On the other hand, all infected feral pigs that developed ataxia recovered fully in 9 to 10 days prior to the end of the study, possibly providing an explanation to this difference. Nevertheless, persistent infection of the tonsils and thymus was demonstrated in the JEV-infected feral pigs. As presented in **Figure 5.8**, similar viral RNA loads were detected in both lymphoid structures of the feral pigs in comparison to those from the domestic pigs (Mann-Whitney U test, $0.222 \leq p \leq 0.690$). Mean viral loads of the thymus was $9.9x10^{0}\pm1.2x10^{1}$ geq-$TCID_{50}$/g for the feral pigs and $1.6x10^{1}\pm2.0x10^{1}$ geq-$TCID_{50}$/g for the domestic pigs. The average viral loads of the tonsil were slightly higher than the thymus, reaching $3.4x10^{3}\pm3.0x10^{3}$ geq-$TCID_{50}$/g in the feral pigs and $9.7x10^{2}\pm1.3x10^{3}$ geq-$TCID_{50}$/g in the domestic pigs.

In summary, the susceptibility, infection course, and clinicopathological outcomes of JEV infection in the feral pig were comparable to what has been previously characterized in the domestic pig challenged with JEV.

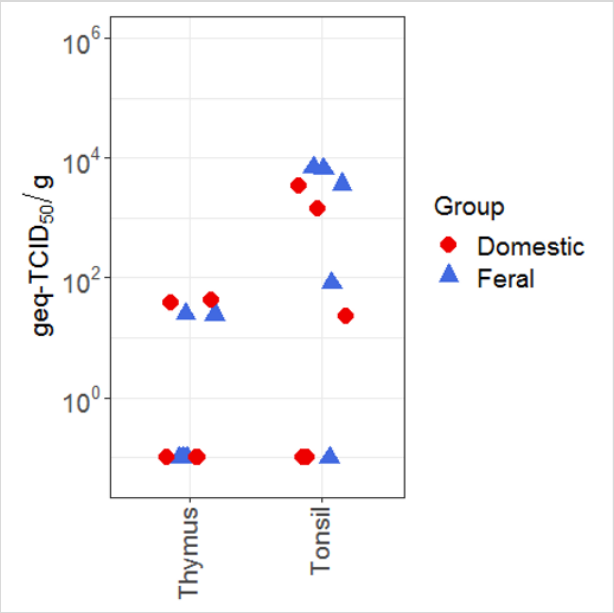

Figure 5.8. Persistent infection of lymphoid tissues collected from JEV-infected feral pigs. Viral RNA loads detected in lymphoid tissues collected at day 28 post-infection from the infected feral pigs were compared to those obtained from the domestic pigs infected with JEV alone in Aim 2. Geq-TCID$_{50}$ = genome equivalent-50% tissue culture infectious dose.

Discussion

While domestic and wild or feral pigs are taxonomically closely related, genetic differences exist between them that could reflect the variation in susceptibility profiles or disease outcomes against a specific pathogen (Conyers et al., 2012; Costa et al., 2012; Lowden et al., 2002). As a result, studying the infection outcome or clinical disease course directly in the animal of interest is justified and provides the direct evidence showing that feral pigs may play a role in the maintenance of zoonotic pathogens. The results from this study demonstrated that feral pigs from North America are susceptible to JEV infection similarly to their domestic counterparts with some minor differences. Using the Sinclair miniature pigs as a model of North American feral pigs, the miniature pigs infected with JEV developed mild to no clinical signs including fever, depression, and hindlimb ataxia of short duration. The clinical disease observed in the infected feral pigs were

comparable to those observed previously in domestic pigs (Park et al., 2018; Ricklin et al., 2016b; Yamada et al., 2004). No demonstratable differences were also observed in the kinetics of viremia, viral titers of nasal secretions, nor in the overall tissue tropism between the feral and domestic pigs after intradermal JEV challenge. The infected feral pigs also exhibited JEV persistence in the lymphoid tissue up to day 28 post-challenge (**Figure 5.8**), demonstrating that they can be used as an infection model for flavivirus persistence.

A significant finding from this study is that the potential amplifying role of feral pigs in JEV transmission was highlighted based on their ability to develop viremia of high magnitude. While the peak viral burden in the serum in both infectious viral titers and JEV RNA loads appeared a day earlier on day 2 post-infection in the infected feral pigs compared to the domestic pigs, the overall kinetics of viremia between the two groups of animals were not statistically different from one another, as summarized in **Figure 5.5**. Additionally, the high seropositive rate of JEV reported in the wild or feral pigs captured in the endemic areas (Hamano et al., 2007; Nidaira et al., 2014; Nidaira et al., 2007; Ohno et al., 2009; Yang et al., 2012a) suggests that mosquitoes that are competent for JEV transmission readily feed on these vertebrate hosts. Therefore, our work and the seosurveillance studies from JE-endemic regions (Hamano et al., 2007; Nidaira et al., 2014; Nidaira et al., 2007; Ohno et al., 2009; Yang et al., 2012a) altogether demonstrates that wild or feral pigs are amplifying hosts of JEV. At the same time, it is important to interpret our results with some caution. While young Sinclair miniature feral piglets were used as representative models of feral pigs of North America, the observations may not be directly extrapolated to the disease pathogenesis of JEV in adult wild or feral pigs in nature. Inoculation of older Sinclair feral pigs could be conducted in the future because age-related difference in susceptibility and clinical course has been demonstrated in feral pigs (Kaden et al., 2004). For

example, young wild boars frequently developed longer viremia of classical swine fever virus whereas the viremia was often short and transient in adult wild boars (Kaden et al., 2004). Additionally, it would be interesting to investigate whether or not the feral pigs are capable of developing secondary viremia or systemic infection with heterologous JEV strains, as observed in domestic pigs experimentally challenged with different JEV genotypes (García-Nicolás et al., 2017), because the findings could potentially impact our understanding of the virus ecology. Nevertheless, the susceptibility of young feral pigs to JEV remains important and relevant as there are continuously stable populations of piglets due to the high turnover rate of feral pigs in nature similar to domestic pigs in swine or pork production.

Among the minor differences in infection outcomes observed in our study, the duration of nasal shedding of JEV RNA was found to be significantly shorter in the infected feral pigs as compared to the infected domestic pigs, as summarized in **Figure 5.6** and **Table 5.4**. However, this finding may be due to differences in methodology. At the same ages (i.e. three-week-old prior to the start of the study), the miniature feral pigs were much smaller in size than the domestic pigs. Due to the size difference, pediatric-sized cotton swabs were used for the duration of the study instead of the regular-sized cotton swabs that were used for the domestic pigs in Aims 1 and 2 to fit the smaller nostrils of the feral pigs for the nasal swab collection. This could result in smaller amount of samples eluted from the swabs, leading to lower amount of virus and higher chance to be undetected even by the sensitive method of RT-qPCR. Regardless, the nasal shedding viral titers that were detected by plaque assay and RT-qPCR were above the levels demonstrated to be infectious by direct oral and/or nasal contact with naïve domestic pigs (Ricklin et al., 2016a). However, it is unknown if vector-free transmission also applies to feral pigs. JEV RNA has only been detected in the serum (Nidaira et al., 2008) and tonsils (Tan et al., 2012) of wild pigs from

JE-endemic regions. No infectious viruses have been isolated from wild or feral pigs as of yet. The ability of JEV-infected feral pigs to transmit the virus through direct nose-to-nose contact with other naïve feral or domestic pigs and vice versa could have significant implications as contact between feral and domestic pigs in outdoor pens is a common occurrence (Miller et al., 2017; Wyckoff et al., 2009). This potential route of transmission should be investigated in the future.

The other difference demonstrated by statistical analysis was the variation in viral burden in certain CNS tissues sampled at day 3 post-infection (**Figure 5.7**). Infected feral pigs had significantly lower viral titers in the piriform cortex and occipital lobe compared to those from infected domestic pigs. However, the viral burden in the rest of the CNS and lymphoid tissues were comparable between the two groups of animals. Overall, the infection and clinical disease course including viremia kinetics, tissue tropism, and viral persistence were similar and indistinguishable when compared between both types of animals, suggesting that this finding could be of minor importance. At the same time, it could also be a reflection of the potential differences in their immune response that could influence the dissemination pattern of the virus in the brain. While domestic and feral pigs have close taxonomic relationship (i.e. both classified under *Sus scrofa*), they are genetically distinct and belong to different subspecies (Sánchez-Cordón et al., 2019; Watanobe et al., 1999). Differences in genetics could reflect in differential susceptibility to disease or infection as genetics play a key role in immune kinetics for diseases. For example, the white blood cell count has been documented to be significantly lower in wild pigs compared to those of domestic pigs at baseline (Tan et al., 2012). There are also reports on how the T cell responses vary between wild and domestic pigs against a specific pathogen such as African swine fever virus (Hühr et al., 2020; Schäfer et al., 2021). Therefore, how a virus disseminates in the host body or gets cleared from the system could be slightly different between the two types of animals.

More data in terms of additional early time points may be necessary to investigate if there is a true difference in the dissemination pattern of JEV in the acute phase of infection between feral and domestic pigs.

Variation in infection or clinical disease outcomes between wild or feral pigs have been observed in other pathogenesis studies, primarily with African swine fever (Hühr et al., 2020; Schäfer et al., 2021; Zani et al., 2018) and classical swine fever viruses (Brugh et al., 1964). For example, while most minipigs and domestic pigs recovered from a moderately virulent strain of African swine fever virus infection, all wild boars succumbed to its infection (Zani et al., 2018). The difference in infection outcome was determined to be primarily due to the variations in T cell responses in wild and domestic pigs. The strong bias in wild pigs for higher regulatory T cells, which can inhibit host antiviral responses, and perforin+ cytotoxic T lymphocytes compared to domestic pigs were identified to be responsible for the more severe inflammation, tissue damage, and ultimately death after challenge with the particular strain of African swine fever virus (Hühr et al., 2020; Schäfer et al., 2021). Similarly, Brugh et al. (1964) demonstrated that wild swine had shorter prodromal periods and faster rates to death from classical swine fever virus infection compared to domestic pigs. However, other studies have demonstrated that classical swine fever virus infection outcomes were comparable between wild and domestic pigs (Depner et al., 1995; Fukai et al., 2020). In addition to host genetic and virus strain differences, inoculum dosage versus animal size (Brugh et al., 1964), internal parasite load (Brugh et al., 1964; Fukai et al., 2020), differences in levels and periods of stress (Sánchez-Cordón et al., 2019), and gut microbiota (Zhang et al., 2020) are some potential factors that could contribute to the observed differences in infection or disease outcome between wild or feral and domestic pigs. Additionally, comparisons of clinical signs are sometimes made difficult by the fact that wild animals tend to conceal disease

by minimizing sickness behavior (Hühr et al., 2020; Tizard, 2008).

Altogether, our study demonstrates for the first time that feral pigs of North America can contribute to the JEV transmission cycle as amplifying hosts. It also highlighted the use of Sinclair miniature feral pigs as relevant viral infection models for JEV infection and disease and for flavivirus persistence. Additionally, the importance of studying pathogens directly in the animal of interest was emphasized through this study. Although they were minor differences, statistically significant variations in certain infection outcomes were identified between the feral and domestic pigs after JEV challenge.

Chapter 6 - Conclusion, final remarks, and future directions

Conclusion and final remarks

Japanese encephalitis virus is a neurotropic flavivirus capable of causing fatal encephalitis and is currently endemic to the Asia-Pacific region (World Health Organization, 2019). Its potential dispersal into new geographic regions is an important public and veterinary health concern, especially into areas where its major players of enzootic transmission cycle (i.e. mosquito, avian, and swine species) may be present. Our work described in this dissertation demonstrated that North American domestic and feral pigs are susceptible to JEV infection via either intravenous or intradermal inoculation. They were also capable of developing pathological outcomes comparable to what has been previously described for JEV disease in pigs and humans (Park et al., 2018; Ricklin et al., 2016a; Ricklin et al., 2016b; Yamada et al., 2004), as presented in **Figure 6.1**. The common pathological and infection outcomes included viremia indicative of transmission potential, viral shedding, systemic infection, and persistent infection. Since no apparent differences were observed in the pathological outcomes of JE using North American pigs, the findings from these studies are relevant to the biology of JEV. Our work was able to address major questions such as the following: (1) Does mosquito saliva affect the pathogenesis of JEV? If so, how? (investigated in Aim 2) and (2) Are feral pigs susceptible to JEV infection? What potential role could they have in JE transmission? (examined in Aim 3). Collectively, our studies have improved the current knowledge of JEV biology.

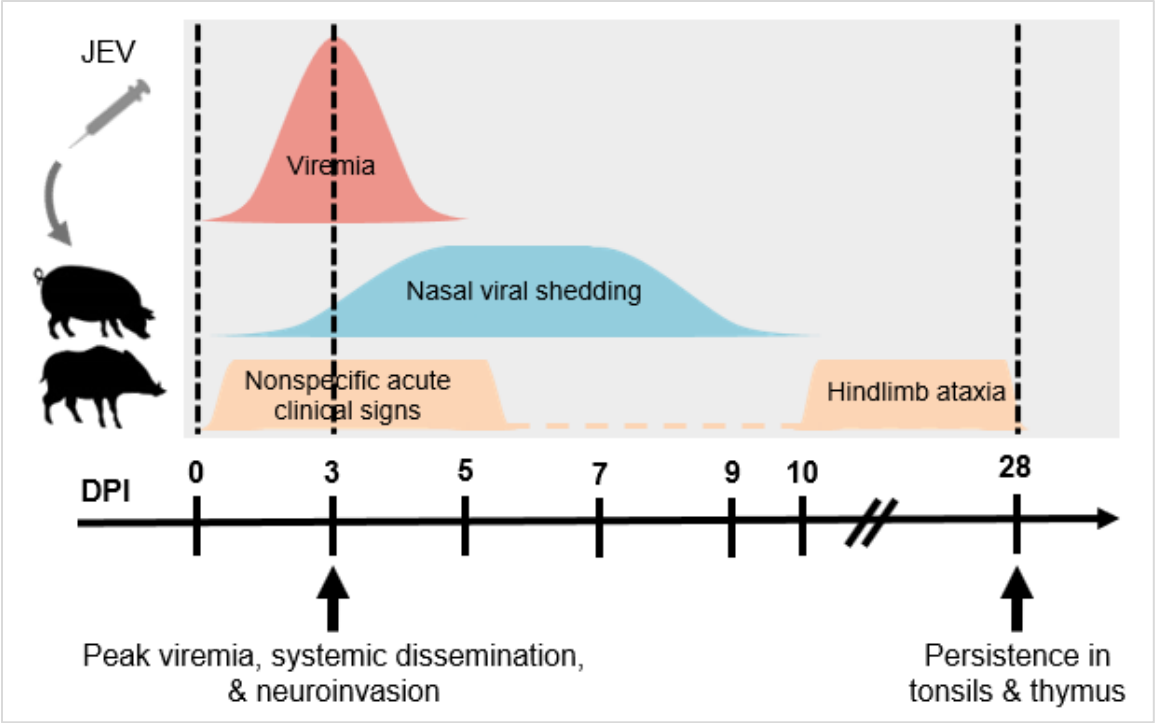

Figure 6.1. Schematic diagram summarizing the results from this dissertation.
JEV = Japanese encephalitis virus. DPI = day post-infection.

The studies described in this dissertation also provide important contribution to the advancement of JEV research. Altogether, they (1) identified appropriate alternative samples applicable in the field for veterinary diagnosis and surveillance, (2) generated an additional animal model for human JE to aid in pre-clinical studies of antiviral or vaccine development, and (3) increased our understanding of how JEV may behave in North America. Ultimately, our work provided additional background knowledge to pursue future research avenues and further our knowledge of JE disease and ecology.

Improvements in veterinary diagnosis and surveillance of JEV

Currently, the diagnosis of JEV in pigs can be based on virus isolation on CNS tissues, viral RNA detection in samples such as blood, brain, placental tissues, and cerebrospinal fluid,

and/or via the detection of JEV-specific antibodies in cerebrospinal fluid or serum samples (World Health Organization, 2019; Yang, 2019). However, the collection of the samples needed to perform these OIE-recommended diagnostic tests are often invasive, time-consuming, and require technical or veterinary expertise. Our studies identified alternative samples that could be used to diagnose and surveil JEV infection in pigs. At the individual level, nasal swabs can be used to collect nasal secretions for the detection of JEV RNA via RT-qPCR, as demonstrated in Aims 1, 2, and 3. At the pen level, collection of oral fluid via rope can be practically implemented to detect the oral shedding of JEV RNA via RT-qPCR (Lyons et al., 2018), as shown in **Figure 6.2**. Although both types of samples are easy and non-invasive to collect, the timing of collection is crucial because viral RNA shedding via nasal secretions or oral fluid can only be detected between 2 to 10 days post-infection (**Figure 6.1** and **6.2**). In addition, it is difficult to estimate how much time has passed since the infection or exposure because infected pigs often develop no to mild nonspecific clinical signs, the most consistent being the elevation of body temperature (Park et al., 2018; Platt and Joo, 2006; Ricklin et al., 2016b; Young et al., 2020). Detection of IgM and IgG antibodies against JEV could provide such estimation. Moreover, the appropriate selection of RT-qPCR assay for JEV detection is crucial because not all available qPCR assays can detect nor differentiate between different genotypes (Lyons et al., 2018). If these diagnostic samples were to be implemented into a surveillance system, weekly collection of nasal swabs or pooled oral fluid would be recommended to maximize its utility but they would first need to be evaluated out in the field.

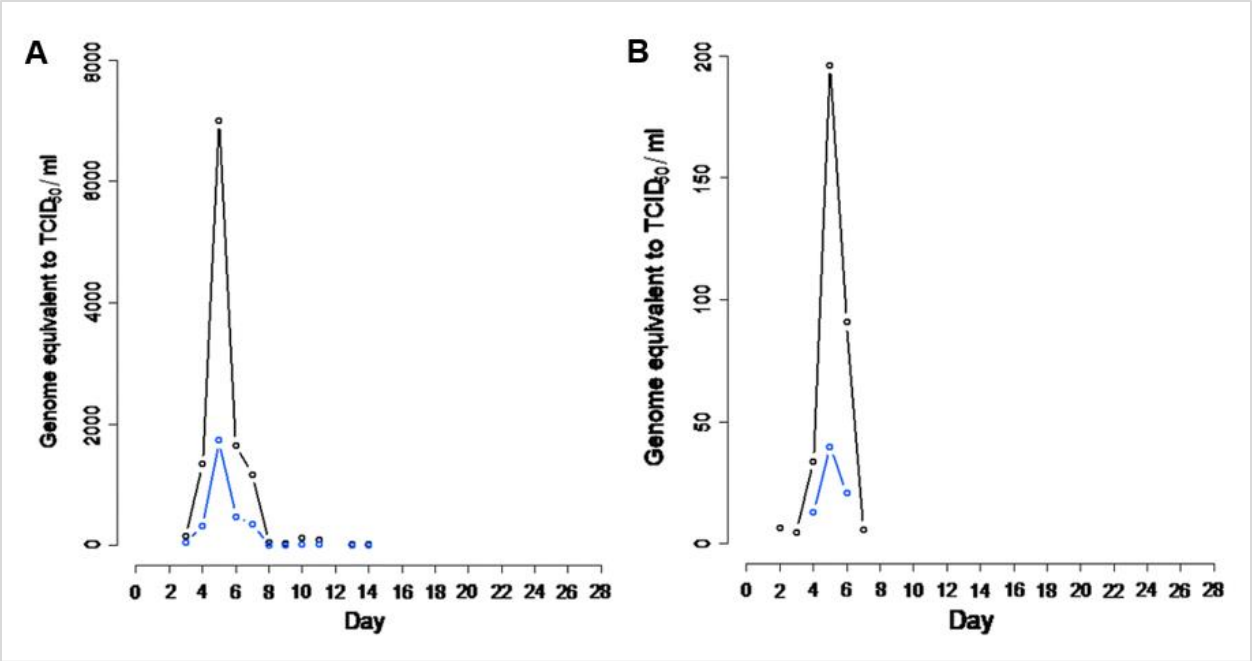

Figure 6.2. Detection of JEV genome in oral fluid collected from intradermally challenged domestic pigs (A) and Sinclair miniature pigs (B) between 0 and 28 days post-infection.

Quantities of JEV genome detected by assay 2 (based on NS5) and assay 3 (based on 3′ UTR) are shown in black and blue, respectively. Only data collected from the samples containing detectable level of JEV genome (Cq value < 34) are shown. $TCID_{50}$ = 50% tissue culture infectious dose. (Image modified from Lyons et al. (2018); Published under the Creative Commons Attribution License).

Domestic pigs are routinely used as sentinel animals to monitor JEV activity in the endemic countries (Cappelle et al., 2016; Di Francesco et al., 2018; Nitatpattana et al., 2011). However, the data demonstrated from our work and recently by others suggest that pigs may not be the most ideal sentinel animal to use for JEV surveillance. Although highly susceptible to JEV infections and a primary player in both the enzootic and epizootic transmission cycles, pigs have the capacity for vector-free transmission of JEV via the oral-nasal route (Ricklin et al., 2016a), leading to a potential misrepresentation of JEV prevalence or risk in the study region (Cappelle et al., 2016; Kading et al., 2019). As a result, support for an integrated surveillance program that involves sampling both the vector and vertebrate hosts may be optimal (Lustig et al., 2018). At a minimum,

sentinel animals should be susceptible to infection, able to seroconvert, and survive the infection (Halliday et al., 2007; Langevin et al., 2001). Ideally, the animals should also not contribute to the arbovirus transmission cycle as amplifying hosts and should not be capable of spreading the infection directly to cage mates via direct transmission because this could lead to misinterpreting the actual risk for mosquito-borne transmission (Halliday et al., 2007; Langevin et al., 2001). An alternate sentinel animal that has been used for JEV surveillance are adult chickens, which meet the minimum criteria, develop low viremia, and are inefficient viral shedders (Auerswald et al., 2020; Nemeth et al., 2012; Yap et al., 2019).

At this time, there is no JEV-specific active surveillance system in North America. Instead, there are existing field and laboratory programs of reporting and surveillance for related flaviviruses that are endemic to the region, such as West Nile and St. Louis encephalitis viruses (Kading et al., 2019). For example, there are state-specific mosquito surveillance programs aimed to monitor select domestic arboviruses (Lustig et al., 2018). There is also a comprehensive national surveillance database platform called ArboNET that passively monitors infections of nationally notifiable arboviruses, such as Eastern equine encephalitis, Powassan, and West Nile viruses, and some travel-associated significant arboviruses, such as chikungunya and yellow fever viruses, in humans, mosquitoes, birds, and other animals (Lindsey et al., 2012; Lustig et al., 2018). These programs potentially could also simultaneously support vector and host surveillance for JEV because some of these nationally monitored, such as West Nile and St. Louis encephalitis viruses, share similar enzootic transmission patterns involving *Culex* species mosquitoes and birds (Diaz-Badillo et al., 2011; Schweitzer et al., 2009). However, because these are related flaviviruses, implementation of JEV diagnostics must be specific enough to differentiate from the other viruses. Cross-reactivity between these closely related flaviviruses is an issue since they also belong in the

same serocomplex. Cases of JEV could thereby go undetected unless JEV-specific tests are utilized routinely. False positive results can sometimes occur for closely related flaviviruses even with the use of the gold standard PRNT (Hirota et al., 2010; Maeki et al., 2019). Cross-reactivity in serological assays can be potentially reduced further through the use of epitope blocking ELISAs (Kitai et al., 2007; Tsai, 1990) or by using novel antigens such as modified E proteins with mutated cross-reactive epitopes (Roberson et al., 2007) and virus-specific recombinant NS1 proteins (Cleton et al., 2015; Mora-Cárdenas et al., 2020).

Pigs as a biologically relevant animal model for human JE

In addition to identifying additional samples for veterinary JE diagnosis, our studies also provide contribution to the development of therapeutic research against JEV. The work in this dissertation demonstrated that young pigs can develop neurotropic disease resembling human JE and identified pathological outcomes that can be markers for protection (Park et al., 2018), making pigs a potential model organism for the pre-clinical development of preventative or therapeutic treatments against JEV infections. As shown in Aims 1, 2, and 3, JEV consistently invaded the brain after peripheral needle inoculation during the acute phase of infection, targeted lymphoid and nervous tissues, and was shed through the oral-nasal secretions as observed in human JEV infections (Bharucha et al., 2018; Ghosh and Basu, 2009; Lyons et al., 2018; Park et al., 2018; Platt and Joo, 2006). Therefore, pigs could function as a biologically relevant model of human JE disease for pre-clinical and translational research. For example, the Yucatan miniature swine model was useful in evaluating the protective efficacy of selected monoclonal antibodies against JEV challenge in a recent study conducted by Young et al. (2020) and others.

Currently, the main animal models to study and characterize human JE disease and develop

therapeutic strategies include non-human primates like rhesus macaques (Gardner and Luciw, 2008; Myint et al., 2014; Myint et al., 1999; Raengsakulrach et al., 1999) and mice (Li et al., 2012; Mathur et al., 1983; Tsuchiya, 1968). The major differences among those established models and the pig are summarized in **Table 6.1**. Both rhesus macaques and mice are good models of fatal JE, but they each have their own limitations. While rhesus macaques are highly susceptible to lethal JEV infection, that disease outcome can only be induced noninvasively by intranasal inoculation (Myint et al., 2014; Myint et al., 1999; Raengsakulrach et al., 1999). Additionally, non-human primates are often prohibitively expensive and associated with regulatory issues that can hinder research progress. On other hand, mice are highly accessible, and various strains are commercially available. Mice are susceptible to neuroinvasive disease through a variety of inoculation routes including intracerebral, intraperitoneal, intradermal, and intranasal (Kimura et al., 2010). However, there is considerable variation in the pathogenesis depending on the inoculation method and they must be young and/or a high viral titer must be used to induce the pathological outcome (Kimura et al., 2010). In pigs, the different modes of JEV infection (i.e. needle [intravenous and intradermal], oronasal, direct contact, etc.) result in similar pathologic outcomes and immune responses (Redant et al., 2020; Ricklin et al., 2016a). Neuroinvasion can also be observed in challenged pigs up to 9 weeks of age (Redant et al., 2020). Pigs offer other advantages over mice. In addition to anatomic and physiologic similarities, pigs have comparable immune system to humans more than humans do with mice (Gerdts et al., 2015; Meurens et al., 2012). Although rodents are evolutionarily more closely related to humans than are pigs to humans, large scale genomic comparisons of immune functions have strongly suggested that pigs and human share more similarities in their immune systems (Bailey et al., 2013; Dawson, 2011; Meurens et al., 2012). Pigs and humans are similar for greater than 80% of the immune parameters analyzed

compared to less than 10% for mice and humans (Bailey et al., 2013; Dawson, 2011; Meurens et al., 2012). At the same time, an advantage of non-human primates and mice over the pig is that they are capable of developing the fatal encephalitis as seen in severe human JE cases while most infections in pigs are often self-limiting, primarily due to the difference in the acute inflammatory responses in the brain (Kimura et al., 2010; Redant et al., 2020). However, kinetics and severity of disease symptoms and pathological outcomes (i.e. duration and magnitude of fever, viremia, viral nasal shedding, and/or neuroinvasion) can be used as markers of infection and disease to thoroughly evaluate the safety and protective efficacy of candidate treatments without requiring an extreme challenge outcome. Pigs are, therefore, an appropriate alternative model for human JE.

Table 6.1. Comparison of animal models of JEV disease.

Non-human primates	Mice	Pigs
▪ Only susceptible to neuroinvasive disease via IN route ▪ Capable of developing lethal infection that resembles fatal human disease ▪ Very expensive and stringent regulations make research practically prohibitive	▪ Must be young or use high titer virus to be susceptible to neuroinvasive disease with IC, IP, ID, and IN routes but there is considerable variation in the pathogenesis depending on the inoculation method ▪ Capable of developing lethal infection that resembles fatal human disease ▪ Limitations for translational research	▪ Susceptible to neuroinvasive disease and develops comparable pathological outcomes regardless of route of challenge (i.e. IV, SQ, ID, IN) ▪ Miniature pig models available for easier handling and husbandry ▪ More similar to the human immune system, anatomy, and physiology than mice are to humans

IN = intranasal. IC = intracerebral. IP = intraperitoneal. ID = intradermal. IV = intravenous. SQ = subcutaneous.
References: (Gardner and Luciw, 2008; Kimura et al., 2010; Meurens et al., 2012; Ricklin et al., 2016a; Young et al., 2020)

Implications of JEV transmission in North America

Overall, the research presented in this dissertation increased our understanding of how JEV may behave in North America. The findings from our work and others demonstrated that all three major players of the JEV transmission cycle (i.e. mosquitoes, birds, and pigs) are now known to be present in North America (Huang et al., 2016a; Huang et al., 2015; Nemeth et al., 2012; Park et al., 2018). Based on a qualitative risk assessment, the likelihood of introduction is small, but the potential still exists (Oliveira et al., 2019). Pathways of entry may include the transport of infected mosquito (via human aid through aircraft or cargo ship) and viremic birds or pigs from JE-endemic regions (via legal or illegal importation, migratory routes, etc.) (Kading et al., 2019; Oliveira et al., 2019). Cases of JEV-infected human travelers coming from the endemic countries have been occasionally reported but humans do not develop sufficient viremia levels to introduce the agent to a susceptible vector (Hills et al., 2019). Nevertheless, once introduced in the United States, an enzootic transmission cycle could potentially become established in North America since all of the key players of the mosquito-vertebrate host cycle are immunologically naïve to the virus, present in the wild, and in close proximity with humans. However, this probability can be variable as the vectorial capacity and host density vary with a high degree of uncertainty depending on the region of introduction (Oliveira et al., 2019). Additionally, both the predictable and stochastic factors influencing an introduction are complex, making risk assessment efforts very challenging and difficult to predict (Kading et al., 2019).

At the same time, there have been several arboviruses that have recently undergone dramatic dispersal events, including African swine fever virus and Zika virus, leading to significant disease burden in the new regions (Kading et al., 2019; Yadav et al., 2016). West Nile virus, a closely related flavivirus to JEV, is an example of an exotic virus that was introduced in the United

Stated in 1999 and became established. The exact mechanism of introduction still remains to be determined but the common culprits (i.e. transport or arrival of infected mosquitoes or birds) have been suggested (Reed et al., 2003; Roehrig, 2013). Based on sequencing and phylogenetic analysis, the New York strain is most closely related to isolates from the 1998 outbreak in Israel but no commerce links between them were made (May et al., 2011; Roehrig, 2013). Nevertheless, once introduced in New York, the virus rapidly spread along the eastern seaboard and from east to west across the country most likely due to the presence of competent mosquitoes and susceptible amplifying migratory avian species (Diaz-Badillo et al., 2011; Reed et al., 2003). Similar to JEV, West Nile virus relies on *Culex* species mosquitoes as vectors and birds as amplifying hosts with humans remaining as dead-end hosts. As such, JEV could potentially exploit the same mechanisms and become established in the region after an introductory event. However, there are some differences between these two viruses that may add more complexity to this theory. For example, JEV is more connected to agriculture. Transmission and thereby most epidemics in Asia are driven by the close association between the amplifying hosts and humans with mosquitoes linking the two types of hosts: (1) domestic pigs and humans in backyard pig farming and (2) water-wading birds and humans in rice paddy fields (Le Flohic et al., 2013; Tsai, 1990). On the other hand, rice production is very limited to certain concentrated locations in the United States (U.S. Department of Agriculture, 2021) and the majority of commercial swine farms are indoor containment facilities (U.S. Department of Agriculture, 2008, 2019; Wyckoff et al., 2009). However, there are a variety of avian species other than the typical water-wading birds that are highly susceptible amplifying hosts of JEV capable of developing high viremia present in North America (Nemeth et al., 2012). Moreover, pigs from indoor facilities can still be exposed to insects including flies and mosquitoes, which have been reported to be responsible for the mechanical transmission of several swine

pathogens between nearby farms (Otake et al., 2001; Otake et al., 2002; Schurrer et al., 2006; Yang et al., 2012b), and there is a large immunologically naïve population of feral pigs available in the wild (Ruiz-Fons, 2017). Therefore, how successful JEV, an agricultural rural pathogen, will be at establishing a permanent transmission cycle in the new territory will most likely vary significantly based on the region of introduction.

Another important factor at play is that the presence of other flaviviruses in North America may also influence how JEV may behave in the environment. Prior to the introduction of West Nile virus, St. Louis encephalitis virus was the only endemic mosquito-borne flavivirus in the United States but was competitively displaced by the emerging virus (Curren et al., 2018; Reisen, 2003). Not only do they share the same enzootic transmission cycle characteristics, but studies have also demonstrated that birds infected with West Nile virus developed sterilizing immunity to St. Louis encephalitis virus while birds previously infected with St. Louis encephalitis virus were still capable of becoming viremic with West Nile virus infection (Fang and Reisen, 2006; Reisen, 2003). Similar results have been demonstrated with JEV. Pigs or birds infected first with West Nile virus and then challenged with JEV were only able to develop low to undetectable levels of JEV viremia and had a booster effect on their already existing antibodies against West Nile virus (Ilkal et al., 1994; Nemeth et al., 2009; Williams et al., 2001). In terms of disease, immunity to West Nile virus reduced the severity of infection and clinical outcome of JEV in macaques (Goverdhan et al., 1992) and mice (Price et al., 1967). These results suggest that pre-existing immunity to West Nile virus may potentially dampen transmission of JEV while simultaneously complicating the serologic diagnosis of JEV (Nemeth et al., 2009). However, approximately 1,500 to 4,000 JE cases are still reported every year in India where both West Nile virus and JEV can be found (Kabilan, 2004; Tiwari et al., 2012). Additionally, JEV has become established in northern

Australia despite the presence of other flaviviruses in that country, such as West Nile and Murray Valley encephalitis viruses (van den Hurk et al., 2019). Coupled with the fact that JEV is a highly neurotropic virus, dispersal of JEV into North America is still a true concern of public and veterinary health importance.

Future directions

Based on data presented in this dissertation, future work could focus on two major topics: improving our understanding of (1) the ecological role of persistence of JEV infection and (2) impact of mosquito-borne transmission in JEV disease in pigs.

First, JEV RNA was consistently detected in the pig tonsils long after the acute phase of infection in Aims 1, 2, and 3. While our studies demonstrated persistent infection at 28 days post-infection, García-Nicolás et al. (2017) showed that JEV can persist in the tonsils even longer, up to 46 days after challenge. Therefore, pigs can be a good model for persistent flavivirus infections. For viruses to establish persistent infection in *in vitro* or *in vivo*, specific host defenses need to be evaded or controlled in order to maintain viral genomes within a small proportion of infected cells (Guo et al., 2018; Mlera et al., 2014). Accordingly, JEV persistence in the pig tonsils may be partly associated with decreased activation of certain immune responses such as IFITM3 (interferon induced transmembrane protein 3), which is considered to be one of the most important IFN-stimulated protein directed against flaviviruses, possibly resulting in the reduced ability of the cells to clear intracellular JEV (Gorman et al., 2016; Xi et al., 2020). Since these findings were based on *in vitro* pig tonsillar epithelial cells, it would be interesting to investigate if they also apply in *in vivo*. Alternatively, the persistent viral genome detected in the pig tonsils can be sequenced to examine whether or not the accumulation of certain mutations is what allows a more efficient

immune evasion and/or if they are primarily persistent defective viral genomes. Nevertheless, the significant biological question that needs to be addressed is whether or not the persistent JEV in the tonsil has the ability to become reactivated. Reactivation of JEV has been possible in studies involving mice and certain cell lines through immunosuppressant drugs (Mathur et al., 1986b), pregnancy (Mathur et al., 1982; Mathur et al., 1986a, b), and allogeneic (Mathur et al., 1986a) or xenogeneic (Sharma et al., 1991) stimulation. Other animal viruses that persistently infect tonsils, such as bovine herpesvirus 1 (Winkler et al., 2000) and porcine reproductive and respiratory syndrome virus (Bierk et al., 2001; Pileri and Mateu, 2016), have been documented to be able to reactivate and cause secondary infections. In a recent study with Seneca Valley virus conducted by Maggioli et al. (2019), stress stimulation in the forms of transportation stress, immunosuppressive drugs, or parturition stress, which are common stressors that affect pigs in commercial farms, were all capable of inducing intermittent viremia and virus shedding that were detectable up to day 60 post-infection. Feral or wild pigs routinely experience additional levels and periods of stress (Sánchez-Cordón et al., 2019). These findings warrant the investigation to determine if pigs, in addition to being efficient amplifiers, can also function as "silent" carriers of JEV, capable of re-shedding and/or developing recurrent infections after initial exposure. Such knowledge can help better define the roles of pigs as an amplifying host in nature.

Second, Aim 2 investigated the impact of mosquito saliva in the disease pathogenesis of JEV through the established method of supplementing the virus inoculation with mosquito SGE. An additional study approach would be to compare JEV infection via SGE supplementation and through direct bites from infected mosquitoes in pigs, especially if pigs are to be used as model organisms for human JE. While these two methods produced comparable infection outcomes and host responses in several pathogenesis studies (Le Coupanec et al., 2013; Moser et al., 2016;

Schneider et al., 2004; Styer et al., 2011), a recent investigation with dengue virus showed they can result in different outcomes important for preclinical evaluation of vaccine or therapeutic candidates (McCracken et al., 2020). For example, rhesus macaques infected with dengue virus with SGE or via infected mosquitoes developed similar local inflammation and immune response at the inoculation site but, while close, the addition of SGE did not recapitulate dengue infection via mosquitoes in terms of viremia kinetics or serum cytokine profile (McCracken et al., 2020). The authors argued that these differences associated with the challenge modality could potentially have great influence over the disease pathogenesis and, consequently, influence the apparent efficacy of a candidate vaccine or treatment strategy. Therefore, it may be necessary in the future to examine the effect of JEV infection via the natural transmission with mosquitoes in pigs in order to potentially improve the challenge model for human JE.

Together, the findings from our studies provide a better understanding of how JEV behaves in its enzootic hosts – the domestic and feral pigs. The knowledge generated from the studies will make a positive impact on public health and the security of U.S. agriculture and livestock. The animal models generated through this dissertation work will provide invaluable aid in the development and implementation of effective countermeasures against this disease and in the research of efficacious therapeutic or prophylactic treatments. Ultimately, data generated through our studies will help support additional future investigations to further our knowledge of JE disease and ecology.

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